Downloaded from orbit.dtu.dk on: May 05, 2023

DTU Library

The quantitative effect of lifetime antimicrobial usage on the abundance of antimicrobial resistance genes in batches of finishing pigs

Dalhoff Andersen, Vibe

Publication date:
2018

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Dalhoff Andersen, V. (2018). *The quantitative effect of lifetime antimicrobial usage on the abundance of antimicrobial resistance genes in batches of finishing pigs*. Technical University of Denmark.

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

The quantitative effect of lifetime antimicrobial usage on the abundance of antimicrobial resistance genes in batches of finishing pigs

---

PhD thesis
Vibe Dalhoff Andersen, DVM
March 2018

National Food Institute, Technical University of Denmark

Supervisors:

Principal supervisor:

Håkan Vigre, DVM, PhD, Associate Professor
Research Group for Genomic Epidemiology, National Food Institute, Technical University of Denmark, Lyngby, Denmark

Co-supervisors:

Leonardo Victor de Knegt, DVM, PhD, Postdoc
Animal Welfare and Disease Control, University of Copenhagen, Frederiksberg, Denmark

Yvonne Agersø, MSc, PhD, Senior Research Scientist
Chr. Hansen Holding A/S, Hoersholm, Denmark

Assessment committee:

Anders Dalsgaard, DVM, PhD, Professor
Food Safety and Zoonoses, University of Copenhagen, Frederiksberg, Denmark

Ulf Emanuelson, Agronom, PhD, Professor
Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

Tine Hald, DVM, PhD, Professor
Research Group for Genomic Epidemiology, National Food Institute, Technical University of Denmark, Lyngby, Denmark

PhD Thesis 2018 © Vibe Dalhoff Andersen
Copyright: Complete or partial reproduction of this publication must include the customary bibliographic citation, including author attribution, report title, etc.
Cover photo: Søren Dalhoff Andersen
Published by: National Food Institute, DTU
Request report from: www.dtu.dk

"However hard we try and however clever we are, there is no question that organisms that have been around for 3 billion years and have adapted to survive under the most extreme conditions, will always overcome whatever we decide to throw at them."

Sir Richard Sykes

Preface

The work presented in this thesis was conducted between October 2013 and March 2018 at the Research Group for Genomic Epidemiology of the National Food Institute, Technical University of Denmark.

The thesis constitutes a part of a larger research project “Vetforlig II”, funded by the Danish Food and Veterinary Administration, aiming at developing a model capable of predicting the abundance of antimicrobial resistance genes in the gut microbiome of finishing pigs close to slaughter, as a response to their antimicrobial usage, for the majority of pigs slaughtered in Denmark. The predictive model will serve as a supportive tool for the Danish authorities to provide guidance for major political and targeted interventions in pig production in Denmark.

The main goal of the thesis was to quantify the effect of antimicrobial usage in finishing pigs on the abundance of antimicrobial resistance genes in their gut microbiome close to slaughter, results that will compose input for the predictive model. Accordingly, the thesis focused first on the development and validation of an optimal method to quantify antimicrobial usage in finisher batches based on register-data and secondly on the quantitative effect of antimicrobial usage on resistance.

Lyngby, March 2018
Vibe Dalhoff Andersen

Content

Preface 5
Content 7
Summary 9
Sammendrag 13
Abbreviations 16
1. Objective and outline 17
2. Introduction 19
2.1 Background 19
2.2 Interventions targeting antimicrobial usage in Danish food production 20
2.3 Antimicrobial usage in Danish pig production 24
2.4 Monitoring antimicrobial usage and resistance 27
2.5 Measuring antimicrobial usage 27
2.6 Measuring antimicrobial resistance 30
2.7 Mechanisms of antimicrobial usage and resistance 30
2.8 Factors affecting antimicrobial usage and resistance 33
2.9 Register-based data 36
3. Objective I 39
3.1 Manuscript I 39
3.2 Supplementary material 52
3.3 Discussion 56
4. Objective II 61
4.1 Manuscript II 61
4.2 Supplementary material 72
4.3 Discussion 78
5. Objective III 83
5.1 Manuscript III 83
5.2 Supplementary material 99
5.3 Discussion 105
6. Conclusions and perspectives 109
Acknowledgements 111
References 113
Appendix A. Additional results to Manuscript I 120
Appendix B. Additional results to Manuscript II 121

Summary

The increased emergence and spread of antimicrobial resistance caused by the widespread use and misuse of antimicrobials are considered among the most harmful threats to global health. It is generally accepted that antimicrobial usage in production animals contributes to the burden of antimicrobial resistant bacteria among humans. Consequently, attention towards antimicrobial usage in production animals has grown immensely during the past decade. In several countries, this has led to the establishing of surveillance systems that monitor trends and changes in antimicrobial usage and resistance in animals over time. In Denmark, the Danish Veterinary Medicines Statistic Program (VetStat) was established. The monitoring systems have in turn been efficiently applied to facilitate interventions targeting antimicrobial usage in animal production. Several epidemiological studies have established that antimicrobial usage and antimicrobial resistance in production animals are closely related. By monitoring antimicrobial resistance over time, it has also been observed that when usage of specific antimicrobials ceased the counterpart antimicrobial resistance decreased. The quantitative relationship between antimicrobial usage and resistance is not as illuminated, as neither the quantification of usage nor the characteristics of the antimicrobials have been fully determined regarding key importance for the selection of antimicrobial resistance. In addition, most studies conducted so far have focused on few indicator bacteria, whereas the bulk of relevant antimicrobial resistance genes might be present in the entire gut microbiota. The recent developments in next generation sequencing allow complete quantification of the abundance of antimicrobial resistance genes in the entire gut microbiome.

Currently, both scientists and authorities are struggling to determine the interventions that will most efficiently achieve the desired reduction in antimicrobial resistance at national level. For this reason, a model capable of predicting the potential effects of any intervention is much needed. Combining precise knowledge of the effect of antimicrobial usage on the abundance of antimicrobial resistance genes with knowledge of antimicrobial usage for larger parts of a population, allows for the prediction of an overall effect of interventions targeting antimicrobial usage in general or for one specific antimicrobial usage only in a population, a country for instance.

The main aim of the thesis was to provide the effect estimates, based on regression analyses of the effect of antimicrobial usage on antimicrobial resistance, for a predictive model. The model was developed for the Danish authorities, as a supporting tool in assessments of the effects of interventions targeting antimicrobial usage on the antimicrobial resistance abundance in the gut microbiome of batches of finishing pigs (finisher batches) close to slaughter at national level.

Objective I.
To assess methods of measure antimicrobial usage in finisher batches close to slaughter independent of rearing site based on register-based data from national databases, by testing the different methods' effects on antimicrobial resistance in the gut microbiome of the batches when antimicrobial resistance is obtained by cultivation and metagenomic (Manuscript I).

Objective II.
To validate the developed method, called “lifetime antimicrobial usage”, by comparing VetStat-records with farm-records. In addition, to assess the influence of an improved lifetime antimicrobial usage on analyses of the effect of antimicrobial usage on the resistance (Manuscript II).

Objective III.
To quantify the effect of lifetime antimicrobial usage in finisher batches on the abundance of antimicrobial resistance genes in their gut microbiome close to slaughter (Manuscript III, not included the predictive model).

Objective I, three methods of calculating antimicrobial usage in finisher batches taking the rearing period and rearing site into account were evaluated by assessing their association with phenotypical resistance and abundance of resistance genes obtained from faeces samples from 10 finisher batches. The antimicrobial usage was calculated relative to the rearing period of the batches as (i) “Finisher Unit Exposure” at unit level, (ii) “Lifetime Exposure” at batch level and (iii) “Herd Exposure” at herd level. A significant effect on the occurrence of tetracycline resistance measured by cultivation was observed for Lifetime Exposure of tetracycline usage. Furthermore, for Lifetime Exposure for the antimicrobial-classes: macrolides, broad-spectrum penicillins, sulfonamides and tetracyclines as well as Herd Unit Exposure for the antimicrobial-classes: aminoglycosides, lincosamides and tetracyclines, a significant effect was observed on the occurrence of their respective genes. No effect was observed for Finisher Unit Exposure. Overall, the study showed that Lifetime Exposure (lifetime antimicrobial usage) is an efficient measurement of usage in finisher batches close to slaughter, i.e. the measurement has a significant effect on the occurrence of resistance, measured by either cultivation or metagenomics.

Objective II, the foundation of lifetime antimicrobial usage; the daily usage, was validated by comparing the transformed data from VetStat with farm-records. Different transformation methods (smoothing) for VetStat-records were examined. Measurement comparisons included accuracy as; completeness and correctness, and precision as; relative difference of the error, correlation with Fisher z transformation and reliability coefficient. The most valid methods of those examined were then used in re-analyses of the abundance of antimicrobial resistance genes in 10 finisher batches from the previous study. Improved accuracy was found when detailed smoothing methods were applied. Although the precision also increased, the effect was not as pronounced, as the usage estimate of all smoothing methods deviated moderately compared with the farm-records. Overall, the smoothing methods underestimated the usage compared with the farm-records. Applying the most valid methods to the 10 finisher batches increased the statistical estimate of model fit for aminoglycosides, lincosamides, and tetracyclines, and decreased the statistical estimate of model fit for macrolides. The statistical estimate of model fit for sulfonamides and broad-spectrum penicillins remained the same. By means of data transformation, VetStat-records can be used to calculate a daily amount of antimicrobial usage per pig, reflecting the true usage accurately and moderately precisely, which is the foundation for calculating lifetime antimicrobial usage

Objective III, 83 farms were randomly selected based on a stratified design related to production-type, annually number of suppliers and annually number slaughtered pigs. The quantitative effect of lifetime antimicrobial usage in the finisher batches on the abundance of antimicrobial resistance genes in their gut microbiome close to slaughter was assessed in regression models. The evaluation of the models' diagnostic plots demonstrated that the assumptions for performing linear relationships were fulfilled, and additional robust regression indicated that the estimated effect was robust against outliers. When the production-type and updated design variables were included in the regression models, it could not be concluded that any of them were important confounders for the effect of lifetime antimicrobial usage. Using linear regression models, significant effects of lifetime antimicrobial usage of one or several antimicrobial classes at dispensing-type level were obtained for each of the seven antimicrobial-classes of resistance assessed. However, two significant effects did stand out, the difference between dispensing-type and the co-selecting effect of peroral macrolides, parenteral and peroral tetracyclines.

This thesis shows that it is possible to describe the quantitative effect of lifetime antimicrobial usage on the occurrence of antimicrobial resistance in real-life conditions. The results are used to develop a predictive model for a large population, in our case most pigs delivered for slaughter in Denmark. A predictive model, where the effect on antibiotic resistance of potential interventions targeting antimicrobial usage, can be tested will be an important tool for the Danish authorities and other stakeholders. However, the predictive model also provides an example of what is feasible and what data will be needed to develop a model that can provide guidance for targeted interventions.

Sammendrag

Den øgede forekomst og spredning af antibiotika resistens er forårsaget af udbredt brug og misbrug af antibiotika. Antibiotika resistens betragtes i dag som en af de mest alvorlige trusler mod global sundhed. Det er almindeligt accepteret, at antibiotikaforbruget i husdyr produktionen bidrager til byrden af antibiotika resistente bakterier blandt mennesker. Opmærksomheden omkring antibiotikaforbruget i produktionsdyr er derfor vokset betydeligt det seneste årti. Det har i flere lande blandt andet ført til etablering af overvågningssystemer, der følger tendenser og ændringer i antibiotikaforbrug og resistens hos dyr. I Danmark blev databasen Veterinær Medicin Statistik Program (VetStat) etableret. Disse overvågningssystemer er efterfølgende blevet anvendt til implementering af målrettede interventioner mod antibiotikaforbruget i husdyrsproduktionen. Flere epidemiologiske undersøgelser har fastslået, at antibiotikaforbrug og resistens i produktionsdyr er tæt forbundet. Ved at overvåge antibiotika resistens over tid er det også blevet observeret, at når brugen af specifikke antibiotika ophører falder den specifikke resistens tilsvarende. Det kvantitative forhold mellem antibiotikaforbrug og antibiotikaresistens er mindre belyst, fordi hverken kvantificeringen eller karakteristika af antibiotika er klarlagt i forhold til betydning for selektion af antibiotika. Hertil kommer, at de fleste hidtil gennemførte undersøgelser har fokuseret på få indikator bakterier, mens størstedelen af relevante antibiotikaresistensgener kan være til stede i hele tarmmikrofloraen. Den nye udvikling inden for DNA sekventeringsmetoder muliggør fuldstændig kvantificering af forekomsten af antibiotikaresistensgener i tarm mikrobiomet.

I øjeblikket arbejder både forskere og myndigheder ihærdigt på at bestemme hvilke interventioner rettet mod antibiotikaforbrug, der mest effektivt vil resultere i ønskede reduktioner af antibiotikaresistens på nationalt plan. Derfor er en model, som er i stand til at forudsige de potentielle resultater af enhver intervention særdeles tiltrængt. At have præcis viden om effekten af antibiotikaforbrug på forekomsten af antibiotikaresistensgener kombineret med viden om antibiotikaforbrug for større dele af en population muliggør prædiktion af den samlede effekt af en intervention rettet mod antibiotikaforbrug generelt eller for specifikke antibiotika i den population, eksempelvis et land.

Den overordnede målsætning med afhandlingen var at beregne effektestimater baseret på regressionsanalyser af effekten af antibiotikaforbrug på antibiotikaresistens til en prædiktiv model. Modellen udvikles til de danske myndigheder, der skal anvende den, som et støtteværktøj i forbindelse med vurdering af effekter af interventioner rettet mod antibiotikaforbrug på forekomsten af antibiotikaresistens i slagtesvins tarmmikrobiom ved slagtetidspunktet. For at opnå målsætningen blev tre formål genereret:

Formål I.
At vurdere metoder til beregning af antibiotikaforbrug for slagtesvin ved slagtetidspunktet uafhængigt af opdrætssted på baggrund af registerbaserede data fra nationale databaser, ved at teste de forskellige metoders effekt på forekomsten antibiotikaresistensgener i slagtesvinenes tarm

mikrobiom, når resistensen er fundet dels ved dyrkning og dels ved sekventering af hele tarmmikrobiomet (Manuskript I).

Formål II.
At validere den udviklede metode til beregning af antibiotikaforbrug ved sammenligning af VetStat-registreringer med besætnings-optegnelser. Derudover at vurdere indflydelsen af en forbedret metode på beregning antibiotikaforbrug i analyser af effekten af antibiotikaforbrug på resistens (Manuscript II)

Formål III.
At kvantificere effekten af livstids antibiotikaforbrug for batch af slagtesvin på forekomsten af antibiotikaresistensgener i deres tarmmikrobiom ved slagtetidspunktet (Manuskript III, the prædiktive model ekskluderet).

Formål I, tre metoder til beregning af antibiotikaforbrug for batch af slagtesvin, der tager opdrætningsperioden og opdrætsstedet i betragtning, blev evalueret ved at vurdere deres sammenhæng til fænotype antibiotika resistens og forekomsten af antibiotika resistensgener i fæces prøver fra 10 batch af slagtesvin. Antibiotikaforbruget blev beregnet i forhold til opdrætningsperiode som (i) "Slagtesvins Eksponering" på enheds-niveau, (ii) "Levetids Eksponering" på batch-niveau og (iii) "Besætnings Eksponering" på besætnings-niveau. En signifikant virkning på forekomsten af tetracyklinresistens, målt ved dyrkning, blev identificeret for Levetids Eksponering for antibiotika-klassen: tetracyklin. Endvidere blev der observeret en signifikant effekt for Levetids Eksponering af antibiotika-klasserne: makrolider, bredspektret penicilliner, sulfonamider og tetracykliner anvendelse samt for Besætnings Eksponering af antibiotika-klasserne: aminoglykosider, linkosamider og tetracykliner på forekomsten af deres respektive resistensgener målt ved DNA sekventering. Der blev ikke observeret effekt for Slagtesvins Eksponering. Samlet set viste studiet, at Levetids Eksponering (levetids antibiotikaforbrug) er en effektiv metode til beregning af antibiotikaforbruget for batch af slagtesvin ved slagtetidspunktet, med en signifikant effekt på forekomsten af resistens målt både ved dyrkning og ved sekventering.

Formål II, livstids antibiotikaforbrug beregnes ud fra det daglige forbrug. Det daglige antibiotikaforbrug blev valideret ved at sammenligne transformerede data fra VetStat med besætnings-optegnelser. Forskellige transformationsmetoder (udjævning) for VetStat-registreringer blev undersøgt. Sammenligning mellem VetStat-data og besætnings-optegnelser udgjorde nøjagtighed som; fuldstændighed og korrekthed, og præcision som; relativ forskel på fejl, korrelation med Fisher z transformation og pålidelighedskoefficient. De mest valide metoder af de undersøgte blev efterfølgende anvendt i re-analyser af forekomsten af antibiotikaresistensgener for 10 batch af slagtesvin fra det tidligere studie. Forbedret nøjagtighed blev fundet, når detaljerede udjævningsmetoder blev anvendt. Selv om præcisionen også steg, var effekten ikke så udtalt, idet forbrugsestimatet for alle udjævningsmetoder afveg moderat i forhold til besætnings-registreringerne. Anvendelse af de mest valide metoder, gav anledning til øgede statistiske estimater for graden af model fit for aminoglykosider, linkosamider og tetracykliner og reducerede for makrolider. De

statistiske estimater for graden af model fit var uforandrede for sulfonamider og bredspektret penicilliner. Ved hjælp af data-transformation er det muligt at anvende VetStat-registreringer til beregning af et daglig antibiotikaforbrug pr. gris, der afspejler det rigtige forbrug nøjagtigt og moderat præcist, hvilket er grundlaget for beregning af levetids antibiotikaforbrug.

Formål III, 83 gårde blev tilfældigt udvalgt på baggrund af et stratificeret design i forhold til produktionstype, antal leverandører og antal slagtede grise årligt. Den kvantitative effekt af levetids antibiotikaforbrug for batch af slagtesvin på forekomst af antibiotika resistensgener i deres tarm mikrobiom tæt på slagtning blev vurderet i regressionsmodeller. Evalueringen af modellernes diagnostiske plots demonstrerede at forudsætningerne for lineære regression var opfyldt, og yderligere robust regression viste, at den estimerede effekt var robust mod ekstreme observationer. Når produktionstypen og de opdaterede designvariabler blev inkluderet i regressionsmodellerne, kunne det ikke konkluderes, at nogen af dem var væsentlige konfundere for effekten af levetids antibiotikaforbrug. Ved anvendelse af lineære regressionsmodeller blev signifikante effekter af levetids antibiotikaforbrug af en eller flere antimikrobielle klasser på dispenseringstype niveau opnået for hver eneste undersøgte antibiotika-klasse resistens. Imidlertid var to signifikante effekter iøjnefaldende, forskellen mellem dispenseringstype og den co-selekterende effekt af perorale makrolider, parenterale og perorale tetracykliner.

Denne afhandling viser, at det er muligt at beskrive den kvantitative effekt af antibiotikaforbrug på forekomst af antibiotikaresistens under virkelige forhold. Disse resultater vil blive anvendt til at udvikle en prædiktiv model for en større population, i vores tilfælde de fleste svin, der leveres til slagtning i Danmark. En prædiktiv model, hvor effekten på antibiotikaresistens af potentielle interventioner mod antibiotikaforbrug kan testes, vil være et vigtigt redskab for de danske myndigheder og andre interessenter. Den prædiktive model giver dog også et eksempel på, hvad der er muligt, og hvilke data der skal bruges med henblik på udvikling af en model, der kan yde vejledning til målrettede interventioner.

Abbreviations

AMR: Antimicrobial resistance

AM(s): Antimicrobial(s)

AMU: Antimicrobial usage

cfu: Colony-forming units

CHR: Central Husbandry Register

DADD: Defined Animal Daily Dose

DDD: Defined Daily Dose

DVFA: Danish Veterinary and Food Administration

MIC: Minimum Inhibitory Concentration

PDD: Prescribed Daily Dose

PCR: Polymerase Chain Reaction

PMD: Pig Movement Database

qPCR: Real-time Polymerase Chain Reaction

TI: Treatment Incidence

TI_{200}: Lifespan Treatment Incidence

VetStat: Danish Veterinary Medicines Statistic Program

Vet: Veterinarian

1. Objective and outline

Antimicrobial resistance is one of the most worrying threats to global health, causing great socio-economic costs, a trend that is expected to continue unabated. Therefore, extensive work is being carried out around the world on trying to curb the otherwise continued emergence and spread of antimicrobial resistance, including risk assessments and predictive models that encompass, the quantitative effect of antimicrobial usage on resistance. However, the quantitative relationship between antimicrobial usage and resistance has not been fully established. Three objectives were generated to fulfil the main aim of the thesis, which was to quantify the effect of antimicrobial usage in finishing pigs (finishers) on the occurrence of antimicrobial resistance in their gut microbiome close to slaughter. The objectives were:

Objective I
To assess methods for measuring antimicrobial usage in finisher batches close to slaughter independent of rearing site based on register-based data from national databases, by testing the different methods' effects on antimicrobial resistance in the gut microbiome of the batches when antimicrobial resistance is obtained by cultivation and metagenomic (Manuscript I).

Objective II
To validate the developed method, called "lifetime antimicrobial usage", by comparing VetStat-records with farm-records. In addition, to assess the influence of improved lifetime antimicrobial usage on analyses of the effect of antimicrobial usage on the resistance (Manuscript II).

Objective III
To quantify the effect of lifetime antimicrobial usage in finisher batches on the abundance of antimicrobial resistance genes in their gut microbiome close to slaughter (Manuscript III, not included the predictive model).

The thesis begins with an introduction yielding an overview of the subjects under study. The introduction presents the background that has led to the current antimicrobial resistance state. The subsequent overview of previous interventions targeting antimicrobial usage aimed at Danish production animals is followed by a description of the pig production, a description of their antimicrobial usage, and the monitoring of antimicrobial usage and resistance in Denmark. A description of methods to measure antimicrobial usage and resistance, the relationship between them and their mechanisms are also included. The introduction ends with an overview of potential risk factors to consider and an outline of the nature of register-based data. The introduction is followed by three sections comprising the three studies performed and their associated manuscript with supplementary material. A closing generic discussion follows each manuscript. The thesis ends with summative conclusions with a brief exposition of future perspectives.

2. Introduction

2.1 Background

The discovering of antimicrobials (AMs) as treatment options for bacterial diseases has revolutionised modern medicine, by reducing the accompanying morbidity and mortality immensely. Since the first discovery of an inhibitory effect of penicillium mould on bacteria, numerous different AM substances have been developed and introduced successively for human and animal medicine (Silver, 2011; Aarestrup, 2015).

In the 1930s and 1940s, sulfonamides and penicillin, respectively, were the first AMs introduced to treat humans with bacterial infections (Levy and Marshall, 2004). Shortly after their introduction in hospital settings, single-drug resistant strains appeared (Levy and Marshall, 2004; Laxminarayan *et al.*, 2013). By the late 1950s, multi-drug resistant strains were detected, however, at that time perceived as a rarity with little health impact (Levy and Marshall, 2004). Similar patterns for evolution of antimicrobial resistance (AMR) have followed all AMs introduced in human and animal medicine, and more worrying. their effective lifespan has decreased over time. Presently, newly developed substances are expected to last only 10-20 years (Levy and Marshall, 2004; Marshall and Levy, 2011; Aarestrup, 2015). The emergence and spread of AMR combined with the lack of novel discoveries at a sufficient rate, increasingly compromise the number of AMs that can be used effectively to treat bacterial infections in humans and animals (Levy and Marshall, 2004; Silver, 2011; Aarestrup, 2015; Laxminarayan *et al.*, 2016).

At the beginning of the 2010s, the World Health Organization named AMR one of the most significant global threats to public health of the 21st century (WHO, 2012). The emergence and spread of AMR is currently devastatingly for society. In 2013, in the European Union, healthcare costs and loss of productivity associated with infections by multi-drug AMR bacteria were estimated to €1.5 billion. Furthermore, these infections caused the deaths of at least 25,000 people (ECDC/EMEA, 2009). During the same year in the United States, the costs related to AMR were estimated to $55 billion and caused the deaths of at least 23,000 people (CDC, 2013). It has been estimated that by 2050, 10 million lives a year will be at risk as a result of infections caused by AMR bacteria, with a cumulative economic cost of US$100 trillion (O'Neill, 2016). By comparison, of 56.4 million deaths worldwide in 2015, 8.8, 1.3 and 0.4 million deaths were caused by cancer, road injury and foodborne diseases, respectively. The threat from AMR is clear, and the world may be heading for a post-antimicrobial era where common bacterial infections once more becomes lethal (WHO, 2014; O'Neill, 2016).

The Swann report from 1969 began addressing a possible association between antimicrobial usage (AMU) in animals and findings of AMR bacteria in humans (Swann *et al.*, 1969). Today, it is generally agreed that resistant strains of *Salmonella*, *Campylobacter* and some *Enterococci* in humans relate to the animal reservoir. In contrast, the actual contribution of AMR found in bacteria of animal

origin, as a burden to human health, is persistently debated (Fey *et al.*, 2000; Marshall and Levy, 2011; Aarestrup, 2015). At the present time, the majority of scientific experts agree that the AMR contribution from food-producing animals is disturbing, and efforts should be aimed at reducing AMU, thereby reducing the emergence and spread of resistance from this reservoir (Aarestrup and Wegener, 1999; Marshall and Levy, 2011; Laxminarayan *et al.*, 2013; Zhang *et al.*, 2013; Aarestrup, 2015; Singer *et al.*, 2016).

2.2 Interventions targeting antimicrobial usage in Danish food production

The introduction of AMs in production animals in the 1950s, substantially improved the treatment of bacterial infections, with great effect on the overall health, welfare and productivity of the animals. Soon after, the growth-promoting effect of AMs were discovered, and usage for this purpose became widespread (Martel *et al.*, 2001).

Based on the Swann Committee recommendation (Swann *et al.*, 1969), the European Council ruled in 1970 that AMU as feed additives was permitted provided the antibiotic did "not endanger animal or human health nor harm the consumer of livestock products" (European Council, 1970). However, as this precaution did not account for bacterial evolutional adaptability, the growth promoter avoparcin for pigs and chickens could be linked to increased findings of *Enterococcus* resistant to the antimicrobial; vancomycin, which at the time was a last resort drug in human medicine (Bager *et al.*, 1997; Wielinga *et al.*, 2014). After the avoparcin usage had ceased, the occurrence of vancomycin-resistant enterococci in production animals decreased (Aarestrup *et al.*, 2001), though the occurrence of vancomycin-resistant enterococci in pigs did not decrease significantly until the use of growth-promotor; tylosin ceased (Jensen and Hayes, 2014). The persistence of VRE in pigs at low levels has been attributed to co-selection due to tylosin resistance genes and to copper resistance genes (Hammerum, Lester and Heuer, 2010).

Battling AMR has high priority in Denmark, and several initiatives aimed at reducing resistance, through intervention targeting AMU, has successively achieved a decline in the usage in food-producing animals, particularly in the pig sector (Wielinga *et al.*, 2014), with an accompanying reduction in AMR (Aarestrup *et al.*, 2001; Agersø and Aarestrup, 2013). Moreover, the reduced AMU has apparently had no long-term negative effect on productivity in pig production (Aarestrup *et al.*, 2010; Aarestrup, 2015) nor on prevalence of lesions at meat inspection as a proxy for an increased occurrence of diseases (Alban, Petersen and Busch, 2015). Several European Union countries, e.g. Belgium and the Netherlands, have implemented similar restrictive and comprehensive AMU interventions within the veterinary sector, and have also experienced reductions in AMR in production animals (Speksnijder *et al.*, 2015; Dorado-García *et al.*, 2016; Callens *et al.*, 2017).

As early as in 1995, using avoparcin as a growth promoter for production animals was banned in Denmark due the negative implications vancomycin-resistant enterococci could inflict on the health-care system (Aarestrup *et al.*, 2001) (Fig. 1). To curb AMU further, two limitations on veterinarians'

(vets') sales of prescribed medicines were introduced in 1995. 1) Practicing vets were not permitted to own a company that distributed prescription medications. 2) Vets could only resell legally bought medicines (from pharmacies) to farmers with a fixed profit. In exchange, vets were given new advisory responsibilities, based on monthly farm visits, to improve animal health and biosecurity, with the adoption of the Veterinary Health Advisory Contract between farmers and vets (Wielinga *et al.*, 2014) (Fig. 1). The Veterinary Health Advisory Contract is mandatory for farms over a certain size (Wielinga *et al.*, 2014). Out of consideration for the farmers, those with contracts are permitted to administer the prescribed AMs themselves between the regular vet visits. In the same year, actions were taken to increase awareness of the "Cascade rule", which lowered the large magisterial production of prescribed chemicals occurring at the time (Fig. 1) (Wielinga *et al.*, 2014). These initiatives resulted in a 40% decline in the AM amounts prescribed for therapeutic usage in production animals (Fig. 1). Of note, by 2000, the agricultural industry in Denmark had voluntarily banned usage of all antibiotic growth promotors in poultry and pig production (Emborg *et al.*, 2001; Aarestrup *et al.*, 2001), which accounted for the decline in AMU occurring between 1997-2000. The EU banned usage of antibiotic growth promotors for food-producing animals on January, 2006 (European Council, 1970) (Fig. 1). In the late 1990s, the Danish Veterinary and Food Administration (DVFA) introduced a detailed guideline, ranking drugs of choice for specified bacterial diseases in pigs, aimed at vets. The guideline is based on the available scientific evidence and changes continuously (DVFA, 2005) (Fig. 1).

The European Union launched the Microbial Threat conference in 1997, as resistance to antibiotics and other AM substances was increasing in human medicine. The conference was hosted by the Danish government in Copenhagen in September 1998 and resulted in five Copenhagen Recommendations (Frimodt-Møller, 2004). In Denmark, as a response to the recommendations, the authorities, research sectors, veterinary sector and the agricultural industry reached mutual consensus on the importance of monitoring AMU in production animals. This led to the establishment of the Danish Veterinary Medicines Statistic Program (VetStat) in 2000, which comprises records on all purchased medicines prescribed by veterinarians for animals (Stege *et al.*, 2003).

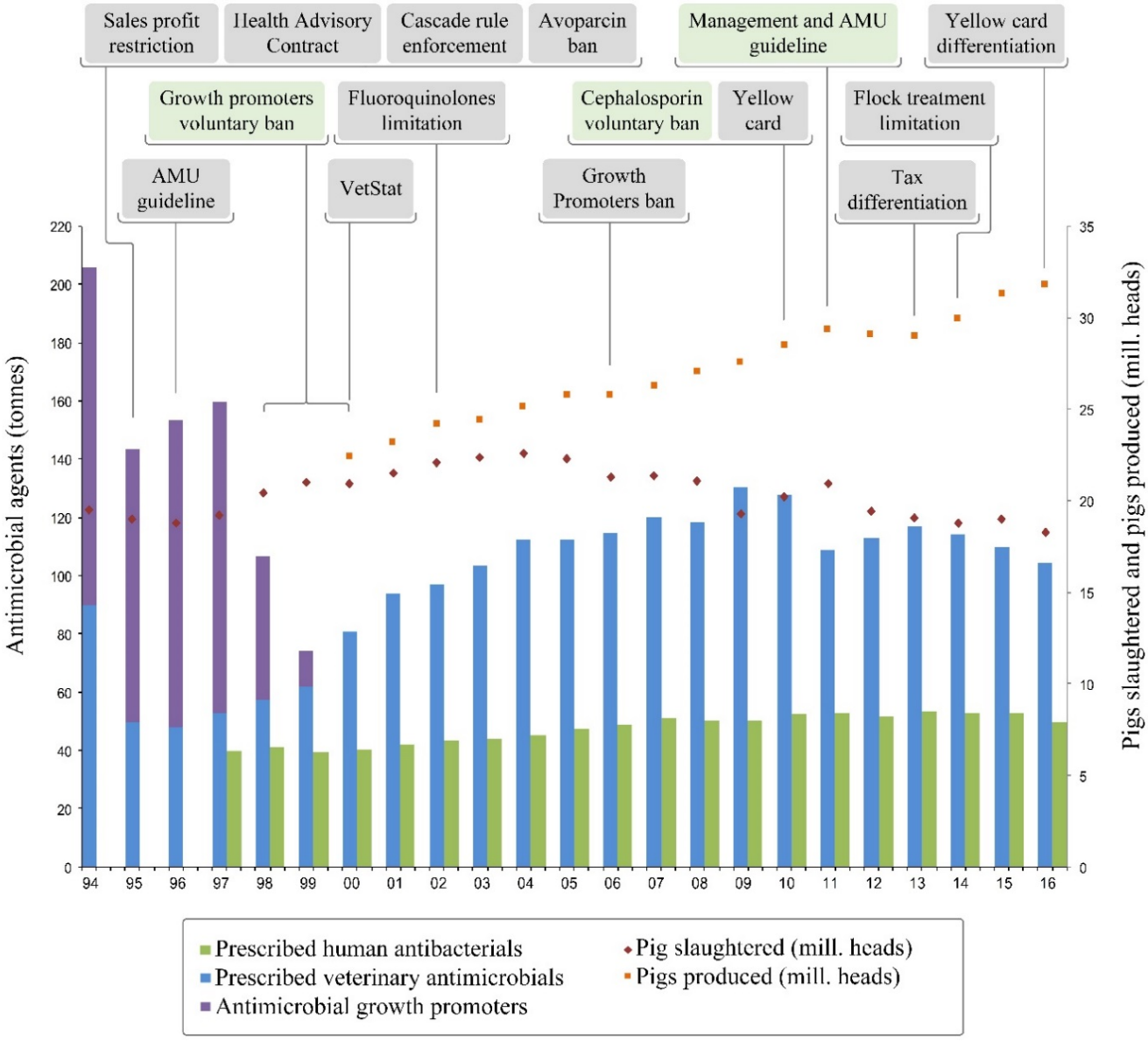


Fig. 1. Prescribed antimicrobial agents in the human and veterinary sector compared with the number of pigs produced and pigs slaughtered in Denmark (SEGES, 2000, 2011, 2016). AMs in the veterinary sector are categorised as either therapeutics or growth promoters (DANMAP, 2017). At the top of the graph, interventions (grey) and voluntary provisions (green) targeted AMs at the year of implementation.

Although the amount of AMU declined from 1994 to 1999, the use in production animals increased by 45% from 2001 to 2009, at which time app. 80% of the prescribed AMs were used in pig production (Fig. 1) (DANMAP 2009, 2010). Part of the increased usage could be explained by a concurrent increase in the production of pigs (Fig. 1). To curb this development, in 2010, the DVFA established the "Yellow Card" intervention, which was based on information from VetStat (Jensen *et al.*, 2014; Wielinga *et al.*, 2014). The Yellow Card intervention was designed to target pig and cattle farmers using high amounts of AMs, by setting national threshold limits for usage. If over a 9-month period a farm exceeds a threshold, the DVFA issues an enforcement notice requiring the farm owner

to bring down the AMU to below thresholds. If the farm does not comply within the granted period, additional enforcement notices are issued (Fig. 2) (DVFA, 2016). The 25% decline in AMU seen after 2010 is assessed to be mainly a result of the Yellow Card initiative (Jensen *et al.*, 2014; Wielinga *et al.*, 2014). The DVFA revises the thresholds every year, based on information from VetStat of changes in AMU (DVFA, 2016).

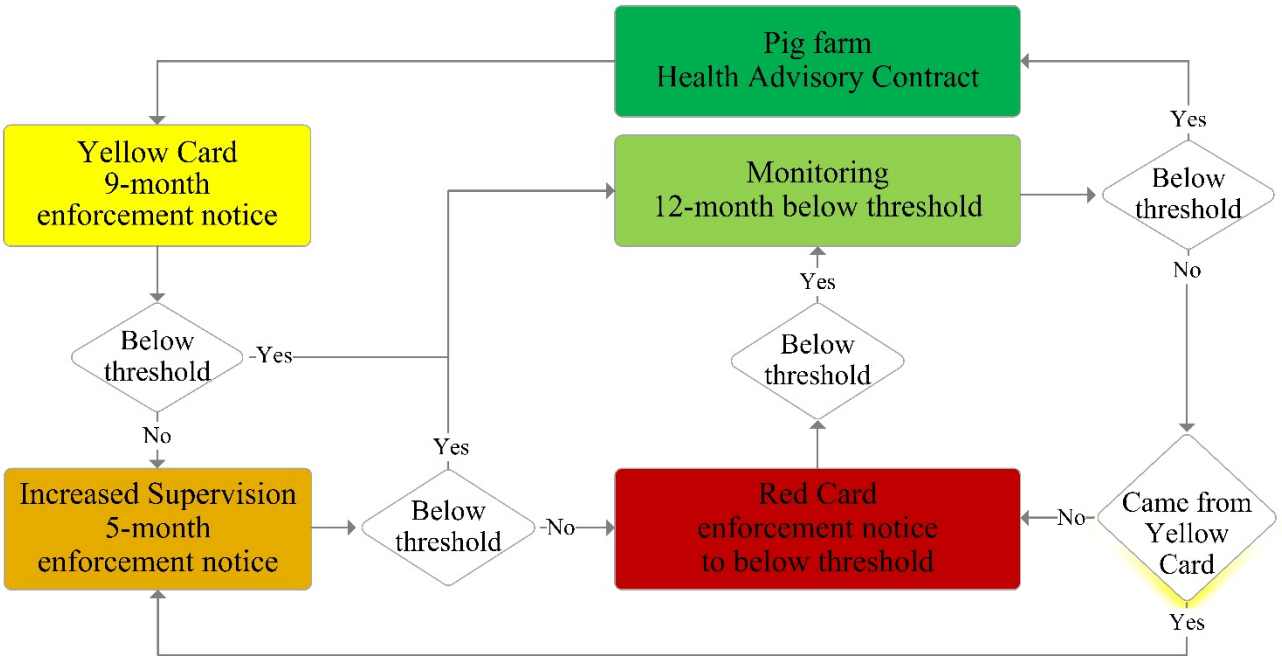


Fig. 2. The Yellow Card (Executive order 179/2014). When a farm confirms exceeding thresholds, a 9-month enforcement notice (Yellow Card) is given to reduce usage in compliance with the thresholds. If the reduction is not achieved during the notice period, the farm is at its own expense, placed under increased supervision and given a 5-month enforcement notice (Increased Supervision) to reduce usage in compliance with the thresholds. If the reduction is not achieved during the notice period, the farm continues under increased supervision at its own expense and is given an additional enforcement notice regarding restrictions on AMU at the farm (Red Card), which is maintained until the AMU complies with the thresholds. When the appropriate reduction is achieved, independent of enforcement level, the farms are monitored for an additional 12 months. However, if the thresholds are surpassed during the 12-month monitoring period, the farm is placed under increased supervision in relation to a previous Yellow Card enforcement notice. Otherwise, the farm is placed at the Red Card level, with increased supervision and AMU restrictions.

In 2010, the pig industry engaged in a voluntary ban of cephalosporin usage in pigs (Agersø and Aarestrup, 2013), and the subsequent year, launched good practice guidelines describing management methods to prevent diseases, and procedures for correct AM administration, aimed at pig farmers (Fig. 1) (SEGES, 2013). To further limit the usage, two interventions were adopted in 2013. Firstly, a differentiated tax favoured simple AMs and vaccines compared with extended substances such as 3th and 4th generation cephalosporins and fluoroquinolones. Secondly, prescriptions issued for flock treatments by water or feed for intestinal and respiratory infections required laboratory verification of the diagnosis on an annual basis. To reduce the usage of critically important AMs for human

medicine (World Health Organization, 2016) in production animals, the most recent initiative involves the modification of the national maximum thresholds in the Yellow Card scheme. In this, the critically important AMs for human medicine have a lower maximum threshold of usage per animal than the AMs of lesser importance for human medicine. Therefore, farms using these classes are prone to meet the maximum thresholds faster (DVFA, 2016). Together, these interventions, particularly those implemented from 2010, appear to have ended the otherwise increasing trend in AMU observed since 1999 (Wielinga *et al.*, 2014). That fact that the AMR levels in Denmark continue to be lower than some of the other EU countries (EFSA/ECDC, 2016) most likely results from the implemented interventions.

Although the level of AMU per produced food animal is low in Denmark, the Danish authorities have committed themselves to ensuring responsible and optimal AMU in the agriculture as part of combatting AMR bacteria threatening public health. However, vets and farmers might not perceive it as equally important. In six European countries (Belgium, Denmark, France, Germany, Sweden and Switzerland), the intentions of vets and farmers to reduce AMU were assessed as their perception of levels of benefits and risks of AMU. Both vets and farmers perceived the benefits as surpassing the risks, however, particularly Danish vets and farmers perceived AMs as more beneficial and less risky than their colleagues from the other countries (Visschers *et al.*, 2016). Visschers *et al*. (2016) argued that the finding might be due to the source of knowledge of benefits and costs, as well as a restrictive policy over a long period, therefore resulting in little scope for further reductions. Moreover, the perceived costs and benefits may be an erroneous perception. Rojo-Gimeno *et al*. (2016) demonstrated that AMU could be reduced by implementing management strategies; biosecurity and vaccines, with a net decrease in costs. The reduction in AMU at the farms related mainly to abandoning regular standard treatments (prophylactic). The discrepancy between vets' and farmers' perception and national AMU policy, combined with the available possibilities within strategic management and increased profit should be taken into account when further interventions targeting AMU are implemented, as vets and farmers are key in the battle against AMR in production animals.

With the prospect of increased demand for animal products due to advances in countries economic status, a concurrent amplification of modern animal production and increase in AMU are expected (van Boeckel et al., 2015). Therefore, in order to enable reduced AMU successfully, targeted AMU interventions with an expected AMR result, combined with altered management strategies, should be employed in food production, through multidisciplinary efforts across sectors.

2.3 Antimicrobial usage in Danish pig production

Since the late 1990s, Denmark has continuously produced an increasing number of live pigs for export and pork production (Fig. 1) (SEGES, 2017). The entire production of pigs reached 31.8 million in 2016, of which 18.3 million finishers were slaughtered in Denmark, and 0.3 million finishers and sows plus 13.2 million 30kg weaners were exported (SEGES, 2017). In the same period,

a vast reduction in the number of farms from 19,823 to 3,294 took place, while the remaining farms increased in size and productivity, a tendency expected to continue in the years to come (Christiansen, 2014; SEGES, 2017).

In 2016, the overall pig production in Denmark took place at conventional farms, while less than 1% of the yearly production of pigs occurs at free-range or organic farms (SEGES, 2017). Of note, some of the differences between these production systems include access to out-doors, available space per animal, feeding requirements, age of weaning and management of antimicrobials, some of which are risk factors demonstrated to potentially affect the amount of AMU and the occurrence of AMR (Mathew *et al.*, 2003; van der Fels-Klerx *et al.*, 2011).

As a consequence of the specialised pig production in Denmark, several different farm systems exist, e.g. integrated production, sows only, sows including weaners, weaners and/or finishers only. The production of a finisher, i.e. rearing from the birth of the piglet to the finisher at time of slaughter, can take place at the same location, at several farms owned by one farmer or at several farms owned by different farmers. Therefore, the rearing pathway of a batch of finishers at time of slaughter could, through complex trade patterns, have passed through numerous farms (Andersen *et al.*, 2017; Birkegård *et al.*, 2017a).

Despite the considerable number of pigs produced per year in Denmark, the biomass of pigs constitutes only 43% of the total biomass of production animals and pets (DANMAP, 2017). The biomass of the cattle population comprises 50% of the total biomass, however, the larger part of the this biomass relates to dairy production, adult animals, whereas the number of pigs produced mostly comprises young animals with a fast turnover rate of approximately 90 and 170 days for 30kg weaners and 100kg finishers, respectively (DANMAP, 2017; SEGES, 2017). The vast number of young pigs in the population may account for the high AMU within the pig production. In 2016, the AMU for pigs constituted 75% of the total usage for animals (Fig. 3). Therefore, the high AMU probably relates to younger pigs being more susceptible to infections combined with the intensive production, early weaning, movements between units in a farm or farms, different environments and mixing of pigs. These factors can cause a suppressing effect on the immune system and an increased spread of diseases (Ekkel *et al.*, 1996; Merlot, Meunier-Salaün and Prunier, 2004; Damgaard, Studnitz and Jensen, 2009; Campbell, Crenshaw and Polo, 2013).

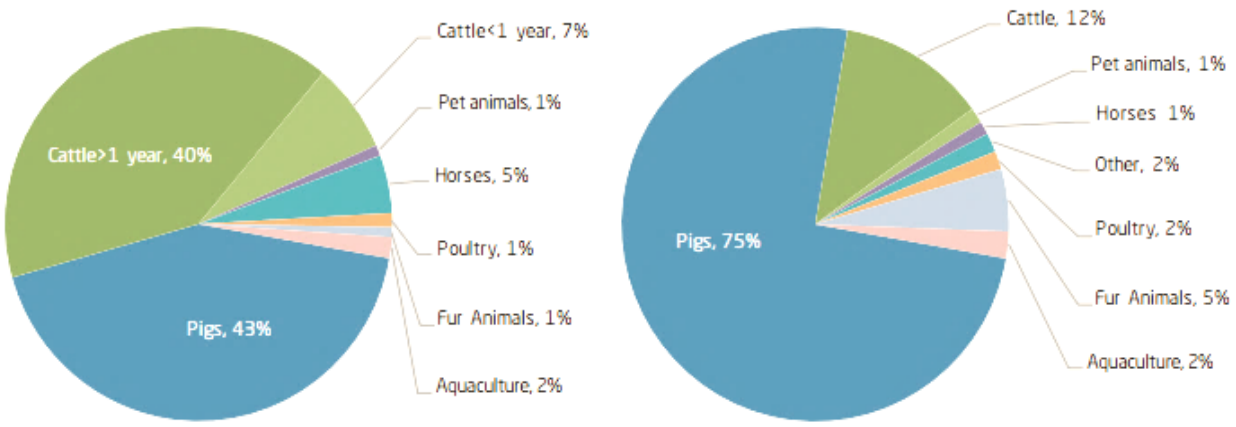


Fig. 3. Live biomass (mill. kg) and antimicrobial consumption (kg) in main animal species, Denmark (DANMAP, 2017)

In line with this, the majority of AMU happens within the age-group; weaners, followed by the age-groups; finishers and sows-piglets-boars (Jensen *et al.*, 2014; DANMAP, 2017). Of the total AMU from 2002 to 2012, 67% was administered perorally either by feed or more commonly by water (Jensen *et al.*, 2014; Dupont *et al.*, 2016). Furthermore, AMs for weaners and finishers were primarily for gastro-intestinal infections, weaners; 75% and finishers; 60%, respiratory infections, weaners; 16% and finishers; 20%, and muscular-skeletal/CNS/skin infections, weaners; 8% and finishers 18%, (Jensen *et al.*, 2014).

The most used antimicrobials for pigs in the past decade, measured as the amount of defined animal daily dose (DADD), have been tetracyclines, macrolides, pleuromutilins and simple penicillins, whereas lesser amounts of lincosamides, extended penicillins, sulfonamides, and aminoglycosides have been used during this period (Jensen *et al.*, 2014; DANMAP, 2017). From 2002 to 2012, tetracyclines, macrolides and pleuromutilins accounted for 80% of the peroral usage and simple penicillins accounted for 39% of the parenteral usage (Jensen *et al.*, 2014).

Usage of the critically important 3th and 4th generation cephalosporin and fluoroquinolones has plummeted since the interventions implemented in 2002 and 2006, respectively. However, from 2009 to 2016, the usage of the critical important colistin increased two-fold to 864kg, probably as an alternative to the concurrent reduction in tetracycline usage, which is used to treat gastro-intestinal infections caused by *Enterobacteriaceae* (DANMAP, 2017). Due to the increasing need for colistin in human medicine and the worrying horizontal spread of colistin resistance genes observed, the risk of colistin usage in veterinary medicine is currently being revised (Rhouma, Beaudry and Letellier, 2016).

2.4 Monitoring antimicrobial usage and resistance

In 1995, the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) surveillance programme was established at the initiative of the Danish Ministry of Health and the Danish Ministry of Food, Agriculture and Fisheries as a coordinated monitoring and research programme. The strength of the programme results from multi-disciplinary collaboration between research sectors and official authorities on animal and human health and their mutual sharing of relevant data from animals, food and humans in order to monitor AMU and AMR across populations (Bager, 2000; Hammerum *et al.*, 2007; DANMAP, 2017). The surveillance provides comparable data on AMU and AMR, and uses these data to follow trends and explore associations between AMU and AMR at regional and national levels. Results are published annually in DANMAP reports (DANMAP, 2017).

VetStat provides the data on AMU in pigs, while the isolates used to monitor AMR are collected from randomly selected healthy pigs at slaughter. The indicator-bacteria *E. coli* is selected to monitor AMR in pigs due to its ubiquitous presence throughout the food chain from the primary producer to the consumer and its ease of gaining and spreading AMR as a response to AMU (Turnidge and Paterson, 2007; DANMAP, 2017). Resistance in *Salmonella* is also monitored for pigs to follow the AMR in zoonotic bacteria (DANMAP, 2017).

The pig slaughterhouses included in DANMAP sampling comprise 95% of pigs slaughtered in Denmark. By means of stratification, the number of samples to collect per slaughterhouse is estimated proportionally based on the number of pigs slaughtered per location compared with the total number of slaughtered pigs from the previous year, in 2016. This secures a representative subset of the entire pig population of pigs at slaughter. When MIC analyses are included, one isolate of each bacterial species per farm per year is susceptibility tested. These procedures ensure that the samples are representative for the occurrence of AMR found in pigs at slaughter, and thus represent an estimate of the occurrence in the entire pig population in Denmark.

2.5 Measuring antimicrobial usage

Different approaches for quantifying AMU at farm level have been presented, such as cost of therapy, total substance weight, defined daily dose (DDD), prescribed daily dose (PDD) and treatment incidence (TI) that uses the DDD in the calculation of AMU (Chauvin *et al.*, 2001). The simplest measurement of AMU at farm level considers solely the weight of the substance used within a specified period for an entire farm. This method does not distinguish between substances with different potency, i.e., the same amount of two substances can be used to treat different number of animals. Therefore, variations in AM usage over time could be the result of product alterations alone. The DDD, PDD, and TI, all take the potency of the substance into account, thereby, providing an approach that measures the relative impact of different substances. Foremost, the choice of method for measuring AMU depends on the subject in study. Subsequently, monitoring of AMU trends over

time requires stability to enable comparison of usages, whereas association studies between AMU and AMR should reflect the level of exposure and the exposure duration (Collineau *et al.*, 2017). Whether the treatment intensity should be calculated as the number of DDD, TI or other comparable measure hereto, is ultimately a matter of tradition and preference. Unless the objective is to compare farms from different countries, then it is paramount to have standardised doses per kg animal and standardised weights per age-group of the species (Bondt *et al.*, 2013; Postma *et al.*, 2015; Taverne *et al.*, 2015)

In 2004 and 2012, the Animal Daily Dose (ADD) and defined animal daily doses (DADD), respectively, were introduced in Denmark as standardised technical measures of AMU (Jensen, Jacobsen and Bager, 2004; DANMAP, 2012). When the ADD is estimated per pig, standard weights for their age-groups are 200kg for sows/gilts/boars including piglets, 15kg for weaners and 50kg for finishers. AMU for piglets is registered in the sows/gilts/boars age-group (Dupont *et al.*, 2016; DANMAP, 2017).

In order to obtain data on AMU, two different approaches are available, obtaining data directly from farms or extracting data from databases. Applying the first will provide exact data, however, it is very time-consuming; due to the hours spent driving back and forth to farms, visiting the farms, and recording non-standardised data to form datasets (Sørensen, Sabroe and Olsen, 1996; Emanuelson and Egenvall, 2014). Consequently, the number of farms included in a study is often determined by these terms. The movements of pigs between farms at different geographical locations further complicates the process of obtaining farm data. For this reason, integrated production is often first choice, when studying associations between AMU during the entire rearing period and AMR in finishers close to slaughter (Postma et al. 2016; Rosengren et al. 2007; Timmerman et al. 2006). In Denmark, integrated production will not apply to a vast proportion of farms delivering finishers for slaughter (SEGES, 2017). When data are obtained by extraction from databases, it is possible to cover larger parts or entire populations. However, the data are not representative of farm usage, but merely a proxy (Sørensen, Sabroe and Olsen, 1996; Emanuelson and Egenvall, 2014). In Denmark, the extraction levels predominantly used in research consist of data at unit, farm and farm-owner level (Emborg *et al.*, 2007; Vieira *et al.*, 2009; Vigre *et al.*, 2010). An AMU calculation restricted to a specified unit (age-group) may lack essential pieces of information when compared with the exposure during the entire rearing period from the birth of the piglet to the finisher at slaughter, because AMU in finisher units is low compared with weaners (DANMAP, 2017). Therefore, the estimation of usage at unit level does not reflect the full exposure during the rearing period. The farm-owner level measures of AMU may include usage of no relevance, i.e. farms and/or units that are not part of the rearing system (Andersen *et al.*, 2017).

The period set for data extraction is most often one year prior to sampling (Emborg *et al.*, 2007; Vieira *et al.*, 2009; Andersen *et al.*, 2015). However, changes in amounts of AMR have been demonstrated to occur over much shorter timespans (Cavaco *et al.*, 2008; Holman and Chénier, 2013), and yearly calculations of AMU might be too crude for association purposes at finisher batch level. Furthermore, in the finisher production, each group of animals (batch) is moved from farrowing to

weaning and then to the finisher unit, primarily on an all-in/all-out basis. Thus, the variation in AMU between batches due to the occurrence of diseases will not be observed, when the usage covers a whole year.

Based on the three databases (CHR, PMD and VetStat), the lifetime AMU for a finisher batch was calculated in steps. First, using the sampling date, the batch was followed backwards through the rearing site(s) on the basis of the national rearing periods, as days in the farrowing, weaning and finisher units, respectively (Jessen, 2015). The number of days was only considered for conventional production, although organic production differs due to the longer period in the farrowing unit. Then, daily amounts of AMU were calculated for all AM-product records *(l)*, during a period *(k)*, in an age-group *(j)* (piglet-sow/boar, weaner or finisher), for all farms *(i)* included in the rearing path of the finisher batch using the formula (Vigre *et al.*, 2010):

$$ADDkg_{ijkl}\ (ADDkg/pig.day) = \frac{product_{ijkl}\ (mg)}{days_k * ADD_l(mg/kg) * pigs_{ijk}}$$

where, *product (mg)* = the recorded amount of an AM product used in an age-group; piglet-sow/boar, weaner or finisher, corresponding to the units in a farm, *days* = the interval in days between the initial recorded date and the subsequent recorded date. The days were estimated first for both dispensing-types as; days between VetStat records at farm and age-group level (Manuscript I), and secondly for peroral as; days between VetStat records at farm, age-group and dispensing-type level, and for parenteral as; days between VetStat records at farm, age-group, dispensing-type and AM-class level (Manuscript II and III). The *ADD(mg/kg)* = the standardised dose per kilogram pig, *pigs* = the number of pigs per age-group *(j)* in a farm unit on any given day obtained from the CHR (Manuscripts I and II), or can be estimated from PMD (Manuscripts II and III) data using the formula:

$$pigs_j = \frac{total\ pigs_j\ (year) * rearing_j}{365\ (days)}$$

where, *total pigs* = the number of pigs produced in a year at a farm i.e. piglets, weaners and finishers, *rearing* = number of days of rearing; 30 days as a piglet, 55 days as a weaner and 85 days as a finisher. The *ADDkg/pig day*, quantifies the daily number of kg-doses per pig in a farm unit (age-group) of an AM product in the period between the initial recorded date and the subsequent recorded date. Hereafter, the daily usage in a finisher batch can be assembled in numerous ways e.g. product- or substance-specific, at age-group or rearing level, for a production period. Alternatively, the daily usage at a farm could be followed over a specified period.

The absolute lifetime AMU was summarised at AM-class and dispensing-type levels by summarising the AMU in the three rearing periods, given by their rearing pathways and adjusted to the proportion of animals being moved from a farm. The lifetime AMU quantifies the total number of ADD kilogram doses used per pig during the entire rearing period of 170 days at AM-class and dispensing-type levels (Andersen *et al.*, 2017). Postma *et al.* (2016) used a similar method of

summarising age-group AMU has been described in a previous study, as a lifespan Treatment Incidence (TI_{200}) calculated for a pig from birth until slaughter using standardised weights; piglets = 2kg, weaners, 7kg and finisher = 35kg.

2.6 Measuring antimicrobial resistance

There is a long tradition of using culture-based methods to determine the occurrence of AMR in research and monitoring systems. The culturing of indicator-bacteria and the determining of their phenotypical resistance include colony-forming units (cfu) counting and single-isolate Minimum Inhibitory Concentration (MIC) determination (EFSA, 2008). The cfu method provides the level of resistance of the bacteria species in a sample by comparing enriched plates with and without a selective AM substance, while the MIC method determines the occurrence of multi-drug resistance at high accuracy in one single isolate. However, none of the methods offer any insight into the underlying mechanism of resistance or the epidemiology within a bacterial population (Munk *et al.*, 2017). The cultivation methods suffer from being time-consuming and labour intensive, but more important due to the limited isolates assessed, the methods provide limited knowledge of the occurrence of AMR across species (Munk *et al.*, 2017)

Several molecular methods focusing on DNA and RNA for characterising and quantifying antibiotic resistance are increasingly being utilised in research. The polymerase chain reaction (PCR) used to detecting AMR in samples is highly sensitive and generates direct information about the DNA sequence of interest within hours at low costs. The quantitative PCR (qPCR) is an expansion of the PCR, i.e. the method encompasses the efficiency of the PCR, providing, in addition, a quantitative estimate of the abundance of the AMR gene targeted. With the recent development in qPCR arrays, the number of genes assessed simultaneously has increased immensely and the method has been beneficially employed to manure and soil samples (Luby *et al.*, 2016; Birkegård *et al.*, 2017). The most recent metagenomics methods are independent of resistance knowledge. Therefore, in a single sample, the collective genes can be sequenced, and using read mapping the AMR can be identified and quantified. The method has already been used for a broad number of sample types, ranging from water, waste-water, human stool and livestock faeces. Due to the usability of the metagenomics methods and the low-cost DNA sequencing technologies within range, these methods may surpass a PCR-based approach. Furthermore, metagenomics methods will provide additional knowledge of e.g. resistance mechanisms, gene location etc. (Luby *et al.*, 2016; Munk *et al.*, 2017).

2.7 Mechanisms of antimicrobial usage and resistance

Long before AMs were discovered, AMR existed in nature, produced by funguses and bacteria as an advantage mechanism to ensure survival (Davies and Davies, 2010; Holmes *et al.*, 2016). Some bacteria e.g. *Actinobacteria*, also contain the corresponding resistance gene, as a self-protection mechanism against the AM they produce themselves (Jiang *et al.*, 2017). Nonetheless, the emergence

of bacteria with corresponding AMR genes as an adaptation defence mechanism against the AMs produced in the natural environment evolved concurrently (Kobayashi *et al.*, 2007; Holmes *et al.*, 2016; Jiang *et al.*, 2017). The group of AMs does not solely comprise the antibiotics; the antifungals, antivirals, and antiparasitics are also included in the AM category. In this thesis, AMs are synonymous with antibiotics only, whether they occur naturally or are produced synthetically.

The different actions of AMs can roughly be categorised as four distinct mechanisms', which include; i) interference with the synthesis of the cell wall or destruction of the cell wall; ii) interference with the DNA/RNA synthesis; iii) interference with the protein synthesis; and iv) inhibition of the metabolic pathway (Fig. 4).

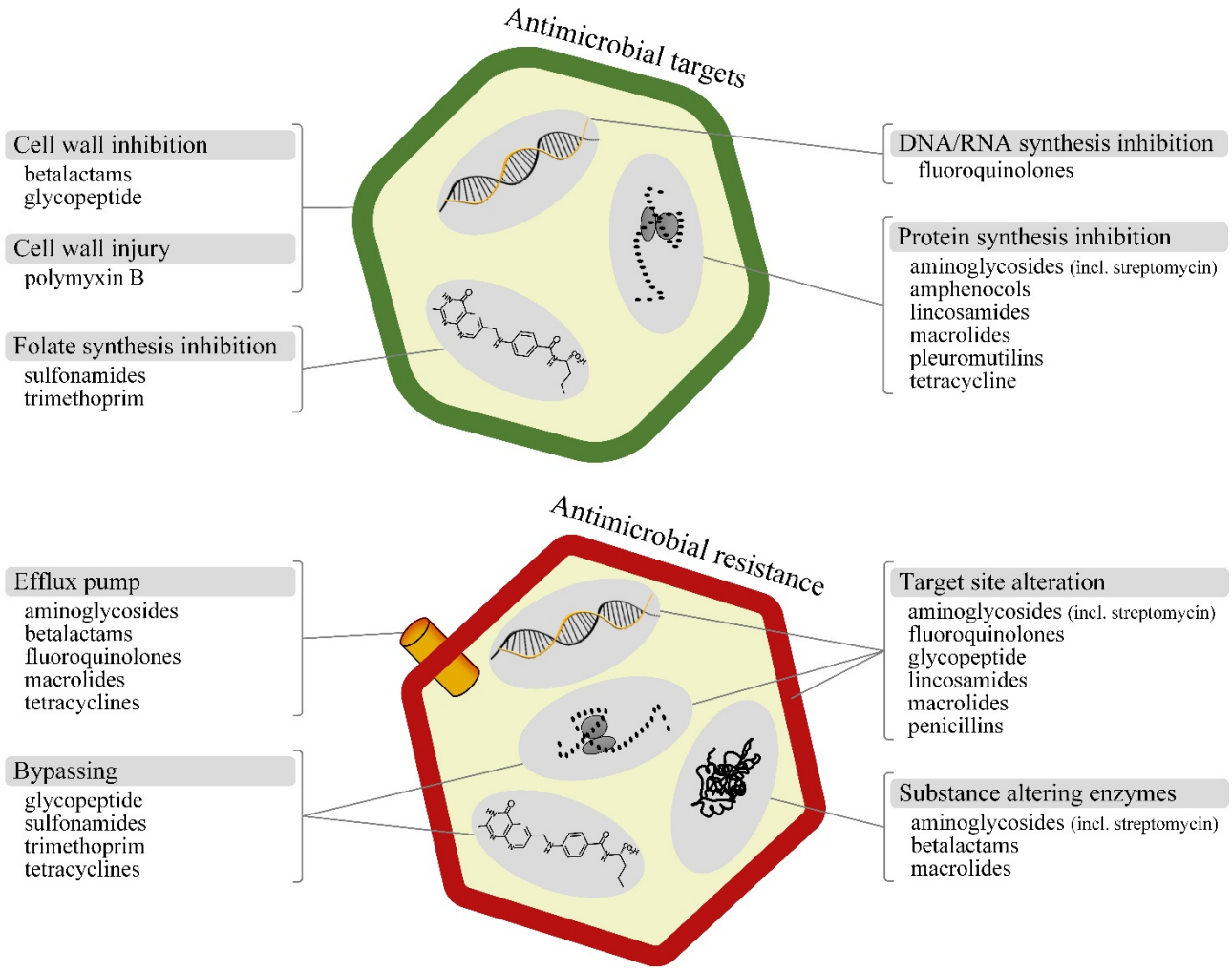


Fig. 4. Mechanisms of action of antimicrobial targets and antimicrobial resistance.

Usage of AMs aims at reducing the occurrence of bacteria that negatively affect human and animal health. However, the widespread use and misuse in modern medicine has reinforced the evolutionary adaptive capacity of bacteria (Darwinism) in order for them to survive in their environment. It is

worth noting that other substances of importance potential causing the emergence and spread of resistance genes in bacteria include heavy metals and biocides, e.g. zinc, copper and quaterium ammonium compounds (Singer *et al.*, 2016), however, the co-selecting effect of biocides is still debated (Wales and Davies, 2015).

The mechanisms of action behind the resistance genes of AMs include; alteration by inactivation or modification of the substance that subsequently becomes inactive, alteration of target site or binding site of the substance that is imperative for the action, since alteration of the metabolic pathway bypasses the action of the substance, reducing membrane permeability (up-take) and causing efflux membrane pumps to actively force the substance out of the bacteria (Fig. 4) (Singer *et al.*, 2016).

AMR can be intrinsic, thus, a natural phenomenon found in the genome of some bacteria species and these resistance genes occur without selection pressure from animal and human AMU (Singer *et al.*, 2016). Whereas acquired AMR is a response to animal and human AMU that develops by means of mutation in the genome, and is then vertically spread by bacteria replication, or happens by acquisition of resistance genes from other bacteria that have the genetic material located on mobile elements, which can then be transferred horizontally. Horizontal transfer includes; transfer through conjugation (contact transfer), transformation (via bacteriophages) or transduction (naked DNA). The mobile elements consist of plasmids, transposons, insertion sequences and integrons. In particular, conjugative plasmids facilitate the horizontal spread of resistance, since plasmids are capable of replication independent of the chromosomal DNA (Aarestrup *et al.*, 2006; Barlow, 2009).

In addition, the AMR level is also fostered by the increased prevalence of multi-drug resistant bacteria, where co-selection promotes the occurrence of resistance genes further. Co-selection is an overall term for co-resistance, where the selection of one resistance gene also promotes the prevalence of other resistance gene(s), through the genomic architecture e.g., combined resistance genes found within plasmids etc., and for cross-resistance, where one resistance gene safeguard from multiple substances e.g. efflux pumps (Cantón *et al.*, 2011; Singer *et al.*, 2016).

Supported by experimental evidence, it is assumed that the carrying of AMR genes comes at a cost (fitness cost) i.e. the ability to survive in a competitive environment is reduced without the selective pressure from the AM. Thus, when an AM is withdrawn the resistant bacteria are out-competed by organisms susceptible to the AM (Wright, 2007; Andersson and Hughes, 2010). However, it has been demonstrated that compensatory mutations can restore the fitness and that some AMR has insignificant or increased fitness (Wright, 2007; Andersson and Hughes, 2010). In an experimental study, it was demonstrated that 21 days after AMU ceased, resistant *E. coli* could still be found in faeces sample from weaners (Cavaco *et al.*, 2008). Therefore, the withdrawals of AMs may not achieve a desired reduction in AMR (Holman and Chénier, 2013, 2015).

Overall, the ability of bacteria to mobilise and transfer resistance genes between bacteria in numerous ways, combined with co-selection from AMs, makes the resistome of the environment, animals and humans, and their mutual connections relevant (Singer *et al.*, 2016; Jiang *et al.*, 2017).

2.8 Factors affecting antimicrobial usage and resistance

The use of AMs is the single most important risk factor in the emergence and spread of AMR in pig production. However, it has been demonstrated that factors related to the management of AMs have an interacting effect on the occurrence of AMR, e.g. usage of several AMs simultaneously and usage at sub-therapeutic doses (Dawson *et al.*, 1984; Akwar *et al.*, 2008; Varga *et al.*, 2009; Looft *et al.*, 2012) (Fig. 5).

Previous studies have demonstrated that the dispensing-type (Varga *et al.*, 2009; Zhang *et al.*, 2013; Burow *et al.*, 2014) and the dose affected the occurrence of AMR (Varga *et al.*, 2009; Zhang *et al.*, 2013). However, these findings have not been supported by other studies involving dispensing-type (Græsbøll *et al.*, 2017) and dose (Burow *et al.*, 2014; Græsbøll *et al.*, 2017) (Fig. 5).

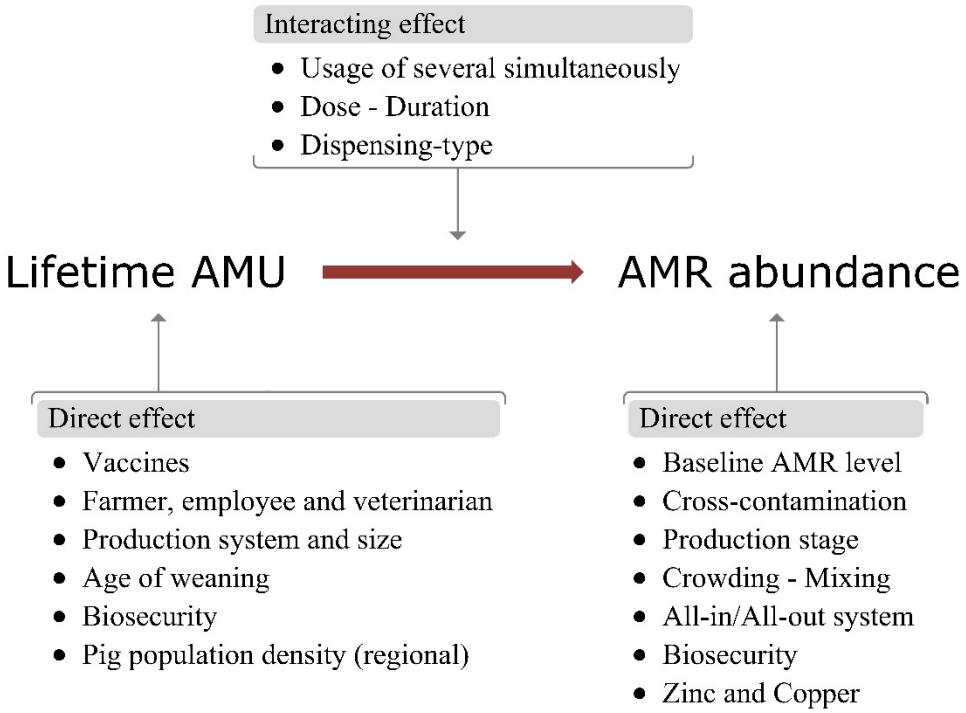


Fig. 5. Causality diagram of antimicrobial usage and antimicrobial resistance in a batch of finishers and the factors with direct and interacting effect.

The AMU history of a farm of origin can affect the AMR in pigs at the subsequent farm (Dawson *et al.*, 1984; Dorado-García *et al.*, 2016). Kietzmann *et al.* (1995) demonstrated that the concentrations of non-metabolised substances and active metabolites in blood of untreated pigs from

one pen depended on whether or not the previous pigs in the same pen had been treated perorally with antimicrobials (Fig. 5).

High number of tetracycline-resistant bacteria in slaughter pigs from farms with no usage have been demonstrated (Dunlop *et al.*, 1998; Vieira *et al.*, 2009), which could be a baseline level as a result of previous usage and maintained by intrinsic or acquired co-selection. Zhang *et al*. (2013) demonstrated high levels of tetracycline resistance in organic farms with no tetracycline usage for years. Taylor *et al*. (2009) demonstrated fluoroquinolone resistance in 19% (*E. coli*) and 54% (*Campylobacter*) at 108 farms with no history of fluoroquinolone usage. Although at low levels, several resistances e.g. chloramphenicol and vancomycin can still be found in faeces samples from finishers even though the AM substances have not been used for several years (Aarestrup *et al.*, 2001; Munk *et al.*, 2017) (Fig. 5).

A pathway for AM contamination from pigs into the surrounding environment is caused by AMU treatments, because 30-90% of the substances will be excreted either non-metabolised or as active metabolites with the manure and urine (Berendsen *et al.*, 2015; Singer *et al.*, 2016). As a result, AMs in waste from treated pigs are disseminated in the pen, and potentially to other pens or the stable. The level of non-metabolised substances and metabolites could be viewed as sub-therapeutic dosages that continuously affect the emergence and spread of AMR bacteria (Berendsen *et al.*, 2015). The AM substances more frequently found in sludge were the less water soluble ones, e.g., trimethoprim, sulfamethoxazole, and doxycycline (Singer *et al.*, 2016) (Fig. 5).

A study comparing farms from Belgium, France, Germany and Sweden demonstrated that lifespan AMU was positively associated with the number of pathogens vaccinated against (Postma *et al.*, 2016, 2016a), and an experimental study demonstrated that the AMU could be reduced by the management strategies; biosecurity and vaccines (Rojo-Gimeno *et al.*, 2016). However, an assessment of vaccination strategies and AMU could not find any relationships between increased usage of vaccines and reduced AMU (Kruse *et al.*, 2017) (Fig. 5).

Studies investigating farm factors related to AMU have demonstrated that integrated productions have lower AMU compared with finisher farms, and the higher the pig population density in a region the higher the farm AMU (Hybschmann *et al.*, 2011; van der Fels-Klerx *et al.*, 2011). Moreover, that higher AMU was associated with relatively smaller farms compared with larger and very large farms (Hybschmann *et al.*, 2011; Vieira *et al.*, 2011). Studies have indicated that vets have a larger influence on AMU at pig farms (Vigre *et al.*, 2010; Hybschmann *et al.*, 2011) (Fig. 5).

The stage of production (piglets, weaner and finisher) has been demonstrated to be a predictor of AMR that could not be explained by AMU alone, with weaners being the age-group with the highest level of resistance (Gibbons *et al.*, 2016). A decrease in AMR during the rearing period has been found in several studies (Dewulf *et al.*, 2007; Hansen *et al.*, 2013). Postma *et al*. (2016) demonstrated that the lifespan AMU (TI_{200}) was reduced in farms with a farrowing rhythm above five weeks and weaning at older ages. As parallels to cross-contamination over time, the tetracycline resistance level

in off-springs has been found to be highly affected by the sow's previous tetracycline usage (Mathew *et al.*, 2005), and the AMR genes from sows have been demonstrated to persist in finishers at time of slaughter (Birkegård *et al.*, 2018). Furthermore, an experimental study showed that transfer of resistance between pens by cross-contamination was possible (Dawson *et al.*, 1984) (Fig. 5).

Studies have demonstrated that mixing of pigs (Dawson *et al.*, 1984; Andraud *et al.*, 2011), crowding and cold stress (Mathew *et al.*, 2003), inside pen hygiene (Dewulf *et al.*, 2007), all-in-all-out system (Schuppers *et al.*, 2005), and lack of rodent control programmes all had an effect on the AMR level (Literak *et al.*, 2009; Vico *et al.*, 2011). Furthermore, AMR bacteria can be transferred between pigs and farmers (Moodley and Guardabassi, 2009; Hammerum *et al.*, 2014). Some studies have demonstrated that heavy metals Zinc (Zn) and Copper (Cu) in soil (Singer *et al.*, 2016; Song *et al.*, 2017) and feed (Hasman and Aarestrup, 2005; Bergenholtz *et al.*, 2009; Cavaco *et al.*, 2010; Cavaco, Hasman and Aarestrup, 2011), by co-selection, affect the occurrence of resistance, and may even exert stronger selection pressure (Song *et al.*, 2017) (Fig. 5).

Increased farm biosecurity measured as transport of animals, removal of manure/dead animals and cleaning/disinfection, have been demonstrated to be associated with increased daily weight gain and reduced AMU, which was presumed to be related to the accompanying reduction of pathogens entering and spreading within a farm (Laanen *et al.*, 2013; M. Postma *et al.*, 2016, 2016a). Backhans *et al*. (2016) demonstrated that the level of biosecurity did not affect AMU, but the individual characteristics of farmer or employees such as age gender and years of experience influences the AMU level (Fig. 5).

Notwithstanding AMs being the most important factor for the emergence and spread of AMR, the number of potential risk factors additionally influencing the occurrence of AMR either directly, through an interacting effect on the AMU and AMR relationship, or through factors affecting the AMU in a pig, is substantial (Fig. 6).

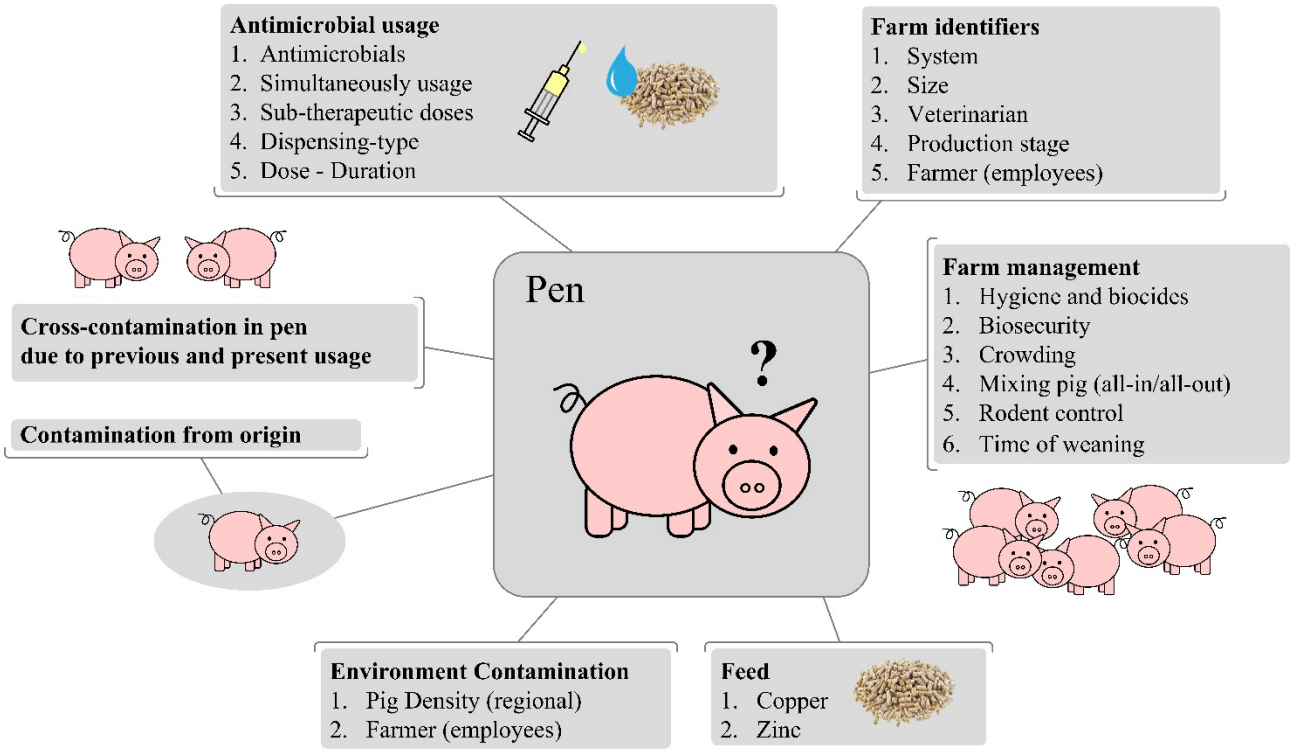


Figure 6. Factors with a potential effect on antimicrobial usage and occurrence of antimicrobial resistance in a pig.

2.9 Register-based data

In epidemiological studies, primary data obtained at farm level are the best source of information, because they have been collected with a specific research question in mind, a hypothesis. In contrast, data extracted from databases, which initially were intended for a specific task, e.g. management, surveillance or control functions, are data collected for other purposes than the initially intended. Thus, the data will not have been collected with a specific research question in mind, and will therefore be considered secondary data. As secondary data has the advantage of often being available in large amounts, the time and cost of obtaining data are less in comparison to collecting the same amount of primary data. Because, secondary data can be obtained for a large part of the population, it is less likely to be affected by selection bias, which may occur when primary data are being collected. The disadvantage of secondary data is that the correctness may vary substantially between sources, and efforts to control or validate it may be beyond the reach of the researcher (Sørensen, Sabroe and Olsen, 1996; Emanuelson and Egenvall, 2014).

With the increased use of register data in veterinary research, it is essential to validate data, in order to work out the extent of data coverage, e.g. the entire population of interest, the registration period time coverage, the accuracy of the data in completeness and correctness, moreover, how precise the data are themselves. These measures will, in turn, demonstrate the quality of the secondary

data, which will be supportive when evaluating the trustworthiness of studies of AMU and AMR relationships using these data (Sørensen, Sabroe and Olsen, 1996; Emanuelson and Egenvall, 2014).

In Denmark, several databases have been established within the veterinary sector, such as the National Central Husbandry Register (CHR), Pig Movement Database, VetStat, National Veterinary practitioners register (VetReg), Laboratory tests register (national mandatory tests), Zoonosis register for *Salmonella* in swine (ZOOR), meat inspection database for cattle and swine, and Swine production data (Houe, Gardner and Nielsen, 2011). These databases are increasingly being used for research purposes due to their completeness at national level.

3. Objective I

3.1 Manuscript I

The association between measurements of antimicrobial use and resistance in the faeces microbiota of finisher batches

V. D. Andersen*, L. V. de Knegt, P. Munk, M. S. Jensen, Y. Agersø, F. M. Aarestrup and H. Vigre.

The National Food Institute, Technical University of Denmark, Lyngby, Denmark

*Corresponding author.

Published in Epidemiology and Infection 2017; 145: 13.
DOI:10.1017/S0950268817001285

CrossMark

Epidemiol. Infect., Page 1 of 11. © Cambridge University Press 2017
doi:10.1017/S0950268817001285

The association between measurements of antimicrobial use and resistance in the faeces microbiota of finisher batches

V. D. ANDERSEN*, L. V. DE KNEGT, P. MUNK, M. S. JENSEN, Y. AGERSØ, F. M. AARESTRUP AND H. VIGRE

The National Food Institute, Technical University of Denmark, Lyngby, Denmark

Received 22 February 2017; Final revision 5 May 2017; Accepted 1 June 2017

SUMMARY

The objectives were to present three approaches for calculating antimicrobial (AM) use in pigs that take into account the rearing period and rearing site, and to study the association between these measurements and phenotypical resistance and abundance of resistance genes in faeces samples from 10 finisher batches. The AM use was calculated relative to the rearing period of the batches as (i) '*Finisher Unit Exposure*' at unit level, (ii) '*Lifetime Exposure*' at batch level and (iii) '*Herd Exposure*' at herd level. A significant effect on the occurrence of tetracycline resistance measured by cultivation was identified for *Lifetime Exposure* for the AM class: tetracycline. Furthermore, for *Lifetime Exposure* for the AM classes: macrolide, broad-spectrum penicillin, sulfonamide and tetracycline use as well as *Herd Unit Exposure* for the AM classes: aminoglycoside, lincosamide and tetracycline use, a significant effect was observed on the occurrence of genes coding for the AM resistance classes: aminoglycoside, lincosamide, macrolide, β-lactam, sulfonamide and tetracycline. No effect was observed for *Finisher Unit Exposure*. Overall, the study shows that *Lifetime Exposure* is an efficient measurement of AM use in finisher batches, and has a significant effect on the occurrence of resistance, measured either by cultivation or metagenomics.

Key words: Antimicrobial drugs, antimicrobial resistance in agricultural settings, cultivation, metagenomic, pigs.

INTRODUCTION

The World Health Organization has declared that antimicrobial resistance (AMR) is one of the most worrying health threats to humans in the 21st century [1], as it adversely affects treatment options in human medicine [2]. Current AMR surveillance is based mainly on passive reporting of clinical diagnoses and phenotypical laboratory results for specific pathogens [3]. However, an approach that provides an insight into the phenotypical resistance may not be representative for the overall occurrence of resistance in the bacterial population it is derived from [3].

Swann *et al.* were the first to raise awareness of a potential link between veterinary use of antimicrobials (AMs) and bacterial AMR in humans [4]. This relationship has since been confirmed by several studies [5–8]. Different methods have been used to measure AMR in the livestock reservoir, including culture-based and molecular methods [3, 9]. Metagenomics sequencing and read mapping have recently been revealed as powerful methods for quantifying AMR in the

* Author for correspondence: V. D. Andersen, Research Group for Genomic Epidemiology, Technical University of Denmark, Søltoft Plads, DK-2800 Kgs. Lyngby, Denmark.
(E-mail: vdan@food.dtu.dk)

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

normal flora of pigs [3]. Quantification of the presence of resistance genes is of relevance when attempting to quantify the contribution from pigs to human pathogens [7, 8, 10].

The association between veterinary use of AM and AMR in bacterial isolates from pigs has been demonstrated repeatedly [11–16] with variations in its occurrence being related to variations in AM use [12, 14–16]. Therefore, less frequent use of AMs in pig farms is a vital factor in reducing the occurrence of AMR in finishers [8, 17–19]. Thus, in order to develop effective tools for interventions and targets for AM reduction, a standardised method is required that closely reflects the dynamics of AMR. As with AMR, which may be measured in several different ways, AM usage may be quantified using different approaches [12, 14–16, 20].

AM use for finishers has traditionally been calculated based on data obtained either from farm records [16, 21–23] or from databases on prescribed medicines: at unit [14, 16], farm [12, 14, 16] or farm-owner level [20, 24]. An AM calculation restricted to a specific unit may lack essential information when compared with the exposure during the entire rearing period from a piglet's birth to the final fattening stage. This is often the case for the finisher unit, where AM use is particularly low [25]. In Denmark, farm-level (integrated) data will not be available for a vast proportion of farms delivering finishers for slaughter [26], as traditional integrated pig production has largely been replaced by multisite pig production where rearing (the farrowing unit → the weaning unit → the finisher unit) takes place on farms at different geographic sites that are owned either by the same farmer or by different farmers [26]. Therefore, the estimation of usage at farm level does not reflect the full exposure during the rearing period. The farm-owner level calculations may include AM use of no relevance if one or more farms are not part of the rearing system.

The periods set for the data extraction in the same studies were mostly 1 year prior to sampling [12, 14, 20]. However, changes in AMR amounts occur at much shorter time spans [13, 16, 27], and yearly calculations of AM use may therefore be insufficiently refined for the purpose of revealing associations at finisher batch level. Furthermore, regarding finisher production, each group of animals (batch) is moved from farrowing to weaning and then to the finisher unit primarily on an all-in/all-out basis. Thus, the variation in AM use between batches due to the occurrence of diseases will not be observed when the calculation of AM use covers a whole year.

By means of the Central Husbandry Register (CHR), the Danish Pig Movement Database (PMD) and the Danish Veterinary Medicines Statistic Program (VetStat), it is possible to calculate an approximation of the AM use per batch based on the rearing period and the rearing site/sites, thus capturing variation in AM use between batches. AMR results for finishers can therefore be obtained at time of slaughter, without consideration for rearing site (s). The objectives of the study were to: (i) develop three different approaches for calculating AM use at finisher batch level, taking into account the rearing period and rearing site, and (ii) compare the association with measured AMR in composite faeces samples from 10 batches of finishers.

METHODS

Data sources

Data on the farms and number of pigs were obtained from the national CHR, where all farms with production animals are recorded [28, 29]. The CHR stores information linked to a farm code (ID), which refers to a specific geographical location and includes, e.g. animal species, the number of animals per age group (sows, weaners, finishers), thus the number per unit on any given day. The pigs owned by a producer (the herd) can be kept at many geographical localities (farms). For the sake of simplicity, all animals owned by an individual producer are referred to as a herd throughout the study even though some herds are kept at many farms [24].

Data on movements of pigs between farms were obtained from the PMD, an integral part of the CHR. The PMD records the number of pigs, date, ID of origin farm and ID of destination farm for each movement [28, 29].

Data on AM use were obtained from VetStat, which contains data on all medicine prescribed by veterinarians for animals. Records are based on veterinarian prescriptions and contain information on active substances, amounts, target species, age groups, diagnosis groups and farm IDs [29]. The age groups in VetStat correspond to the age groups in the CHR, and the units, sows, weaners and finishers on a farm. In order to produce comparable data across records, active compounds were converted into a unit measuring how many kilograms of pig could be treated per

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

day – Animal Defined Daily Doses per kilogram (ADDkg) [30].

Study design

The study design used was first described by Munk *et al.* [3]. In brief, based on the average sizes for sow and finisher farms in 2009 [31, 32], herds with more than 500 sows and a production of at least 5000 finishers annually were selected using the CHR and PMD. In total, 376 herds met these criteria. From VetStat, the total amounts of AM and tetracycline used in these herds from June to November 2013 were calculated as ADDkg and adjusted according to the number of animals in the herds. To cover a wide span of AM use, the 376 herds were ranked, and the owners of the top and bottom 10% AM and tetracycline use quantiles were invited to participate consecutively until five herds within each quantile had accepted. Each of these 10 herds was located between one and seven farms. In total, 23 herds from the top quantile and 15 herds from the bottom quantile were invited.

In the finisher units of the 10 herds, composite pen-floor samples were collected consisting of single samples from 30 randomly chosen pens within the section(s) with finishers weighing between 80 and 100 kg (in Denmark, pigs are delivered for slaughter weighing 100–105 kg) [3,9]. For each herd, the group of pigs from which samples were collected represents a finisher batch. For each of the finisher batches, the 30 single faeces samples were pooled together, resulting in one pooled sample per batch. Sampling took place from March to June 2014.

Measurements of AM use

For each batch of finisher, three quantitative usage measurements of the AM classes: aminoglycoside, lincosamide, macrolide, broad-spectrum penicillin, sulfonamide and tetracycline were calculated. They were based on the day of sampling, as the start from which backward assessment of time periods in the rearing site(s) were established. The measurements varied in terms of exposure, as explained below.

Danish national averages for pig production productivity for 2014 were applied for the rearing periods per unit (in days) [33–35], resulting in 25, 50 and 85 days in the farrowing (piglet), weaning (weaner) and finisher units (finisher), respectively. Starting with the finisher batches and the day of sampling, the PMD was used to trace the movements of the batches from sampling site back to birth site. Figure 1 shows the pathways of the 10 finisher batches.

Each farmer in the study had a health advisory contract with an individual veterinarian in accordance with Danish legislation. These contracts permit veterinarians to prescribe AMs for treatment of plausible diseases diagnosed at a farm visit for the subsequent 35 or 63 days (finishers only) at which point the next visit must take place. Therefore, taking into account the rearing period of 160 days, and the maximum period a prescription may cover (63 days), data from VetStat on prescribed AMs 9 months prior to sampling were extracted for the 22 farms comprising the finisher batches' pathways from birth to sampling site (Fig. 1).

AM exposure was calculated for the classes: aminoglycoside, lincosamide, macrolide, broad-spectrum penicillin, tetracycline and sulfonamide, as these are most commonly used in Danish pig production [25]. By adopting the method used by Vigre *et al.* [24], the daily amounts of the six AM classes used were calculated for sows, weaners and finishers in each of the 22 farms based on two assumptions. First, as veterinarians are permitted to prescribe for potential diseases for 35 or 65 days, the interval between two prescriptions was used to calculate the daily use for each prescription of AM, assuming that an average amount of the prescribed AM was used on each day of the interval. The calculations were subsequently added together per day per AM class. Second, if an interval was shorter than 7 days, the following prescription date was used to set the number of days for the interval.

Thus, the daily use of an AM was calculated as an average daily use per farm (F) per age group/unit (U) and adjusted in accordance with the number of pigs at risk per day per unit on each farm, as:

$$\mathrm{ADDkg\ day}_{\mathrm{AM}\_F\_U} = \frac{\mathrm{ADDkg}_{\mathrm{AMprescribed}\_F\_U}}{\mathrm{days}_{\mathrm{prescription\ interval}\_H} \times \mathrm{pigs}_{\mathrm{day}\_F\_U}},$$

where $\mathrm{ADDkg}_{\mathrm{AMprescribed}\_F\_U}$ = the prescribed amount per unit per farm measured as ADDkg, $\mathrm{days}_{\mathrm{prescription\ interval}}$ = the interval in days between the day of the initial prescription and the day of the subsequent prescription and $\mathrm{pigs}_{\mathrm{day}\_F\_U}$ = the number of sows, weaners or finishers on any given day. The number of sows substituted the number of piglets, as the latter is not registered in the CHR.

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

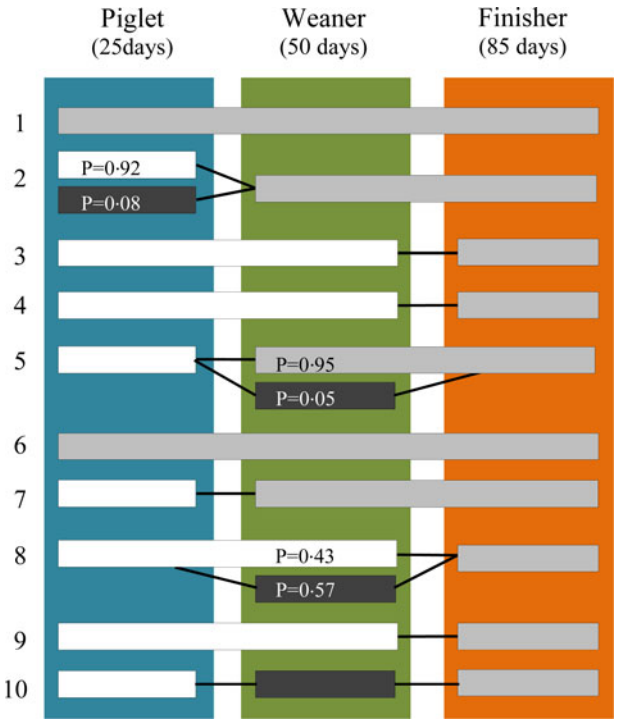


Fig. 1. The rearing pathway of each of the 10 finisher batches from birth site to finisher site compared with the day of sampling. The 10 horizontal bars depict the movements of the batches. A colour shift in a bar denotes that a farm has a different geographical location to the farm where sampling took place and *P* denotes the proportion of pigs being moved. Bars without *P* are equal to one. Numbers 1–5 denote the initially high users and 6–10 depict the initially low users. The three vertical coloured bars represent the assumed days of exposure to AM in the: sow-piglet (farrowing) unit, weaner unit and finisher unit.

The number of ADDkg day $_{\mathrm{AM\_}F\_U}$ was then summarised per day for each AM class (AMc):

$$\mathrm{ADDkg\ day}_{\mathrm{AMc\_}F\_U} = \sum_{\mathrm{AM}=1}^{n} \mathrm{ADDkg\ day}_{\mathrm{AM\_}F\_U}.$$

Based on the finisher batches' rearing periods in days: days 1–85 in the finisher unit, days 86–135 in the weaning unit and days 136–160 in the sow unit, where day 1 corresponds to the day of sampling (Fig. 1), the number of ADDkg day $_{\mathrm{AMc\_}H\_U}$ was summarised for each rearing period (*R*) per unit, and adjusted to suit the proportion (*P*) of animals being moved from a farm (Fig. 1):

$$\mathrm{ADDkg}_{\mathrm{AMc\_}R\_U} = \sum_{\mathrm{date}=1}^{n} \mathrm{ADDkg\ day}_{\mathrm{AMc\_}F\_U} \times P.$$

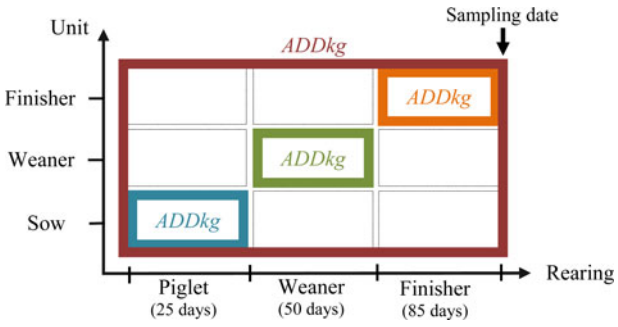


Fig. 2. The exposure measurements of AM usage. The orange square applies to *Finisher Unit Exposure* and therefore comprises the AM usage in the finisher-rearing period in the finisher unit. *Lifetime Exposure* applies to the orange, green and blue squares, and therefore comprises the AM usage in the piglet-rearing period in the sow unit, the weaning-rearing period in the weaning unit and the finisher-rearing period in the finisher unit. The red square applies to *Herd Exposure*, and therefore comprises AM usage throughout the entire rearing period in all units.

For each AM class, three measurements of exposure were calculated, given the rearing pathways, the rearing periods (Fig. 1) and the unit(s) in each farm (Fig. 2).

$$\mathrm{Finisher\ UnitExposure}_{\mathrm{AMc}} = \mathrm{ADDkg}_{\mathrm{AMc\_Finisher\_Finisher}}$$

$$\mathrm{Lifetime\ Exposure}_{\mathrm{AMc}} = \mathrm{ADDkg}_{\mathrm{AMc\_Piglet\_Sow}} + \mathrm{ADDkg}_{\mathrm{AMc\_Weaner\_Weaner}} + \mathrm{ADDkg}_{\mathrm{AMc\_Finisher\_Finisher}}$$

$$\mathrm{Herd\ Exposure}_{\mathrm{AMc}} = \mathrm{ADDkg}_{\mathrm{AMc\_Piglet\_All}} + \mathrm{ADDkg}_{\mathrm{AMc\_Weaner\_All}} + \mathrm{ADDkg}_{\mathrm{AMc\_Finisher\_All}}.$$

For information on the units per farm included in the exposure measurements in the rearing period of the 10 finisher batches, see Figure S1 in the Supplementary data. For information on the obtained exposure variables, see Table S1 in the Supplementary data.

AMR measurements

In this study, the cultivation and metagenomic results of the pooled samples from 10 pig herds described by Munk *et al.* [3] were used as the AMR results.

Cultivation

In brief, for each pooled sample, faeces was suspended in isotonic saline prior to serial dilution. Aliquots of

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

dilutions were plated onto selective and non-selective LB and MacConkey plates to quantify aerobic bacteria and *Escherichia coli*, respectively. Selective plates contained 8 mg/l tetracycline (T3383 tetracycline hydrochloride, former: Sigma-Aldrich, current: Thermo Fisher Scientific, Roskilde, Denmark) in LB and 16 mg/l ampicillin (A9393 Ampicillin, former: Sigma-Aldrich, current: Thermo Fisher Scientific, Roskilde, Denmark) or tetracycline in MacConkey. All assays were performed in triplicate. For each triplicate set, a weighted average of resistance proportion was calculated based on cfu counts per dilution [3]. For information on the obtained outcome variables, see Table S1 in the Supplementary data.

Metagenomics

In brief, AMR genes for the classes: aminoglycoside, lincosamide, macrolide, β-lactam, sulfonamide and tetracycline were obtained using whole community sequencing (WCS), and measured as reads per kilobase reference per million (RPKM). For information on the genes within each AMR class, see Table S2 in the Supplementary data.

In order to compare the 10 faeces samples, the raw read counts were normalised to the size of the dataset for each AM class with the following formula:

$$\text{Reads per kilobase reference per million}_{\text{AMc}} = \left(\frac{n}{N(l-(i-2m))}\right)10^6 R \times 1000 \text{ bp},$$

where n = number of mapped reads, N = total number of reads, l = gene length, i = insert size, m = minimum mapping length, R = reads and bp = base pair.

The normalisation takes into account the fact that the pooling and sequencing of several indexed samples produces varying DNA library sizes, resulting in comparable RPKM values and independence of sequencing depth. For information on the obtained outcome variables, see Table S1 in the Supplementary data.

Data analyses

The quantitative effect of broad-spectrum penicillin and tetracycline use, measured as *Finisher Unit Exposure*, *Lifetime Exposure* and *Herd Exposure*, on ampicillin and tetracycline resistance obtained by cultivation was calculated using simple linear regression, and as a measure of model fit, the coefficient of determination (R^2) was applied. The quantitative effect of exposure for the AM classes: aminoglycoside, lincosamide, macrolide, broad-spectrum penicillin, sulfonamide and tetracycline, measured as *Finisher Unit Exposure*, *Lifetime Exposure* and *Herd Exposure*, on similar AMR gene abundance was calculated using simple linear regression, and as a measure of model fit, the coefficient of determination (R^2) was applied. The assessment of homoscedasticity was performed by visual inspection of the plots, including measured values and regression lines.

WPS Workbench, Version: 3.1.1.0.0, and Microsoft Excel 2010 were applied in data processing, and data analyses were performed using R, version 3.

RESULTS

The three different quantitative measurements of total AM use presented in Figure 3 show use within the applied rearing periods. The *Lifetime Exposure* is lower than *Herd Exposure*, as the former consists of a part of the latter. The difference is caused by the level at which data were obtained; the *Lifetime Exposure* was obtained at rearing batch level, while the *Herd Exposure* was obtained at rearing herd level. A similar pattern applies to the *Finisher Unit Exposure* compared with the *Lifetime Exposure*, since the latter was obtained at batch level, whereas the former was obtained at unit level. Figure 4 shows the distribution of the intervals in days between prescriptions. Most of the intervals are between 24 and 45 days, with fewer between 7 and 21 days. The short prescription intervals were observed mainly in the initially high-user farms. Of these intervals, two-thirds were due to a subsequent prescription of the same AM, with the remaining third caused by a different prescribed AM.

Association between AM use and phenotypical measured resistance

Figure 5 shows the regression models with 95% confidence intervals of the quantitative effect of *Lifetime Exposure* for broad-spectrum penicillin and tetracycline use on the average proportion of ampicillin and tetracycline-resistant *E. coli* (MacConkey), respectively, and of *Lifetime Exposure* for tetracycline use on the average proportion of tetracycline-resistant aerobic bacteria (LB). The β-values, P-values and R^2 values for the regression analyses are shown in the same figure. The regression analyses showed only a significant effect of *Lifetime Exposure* for tetracycline

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

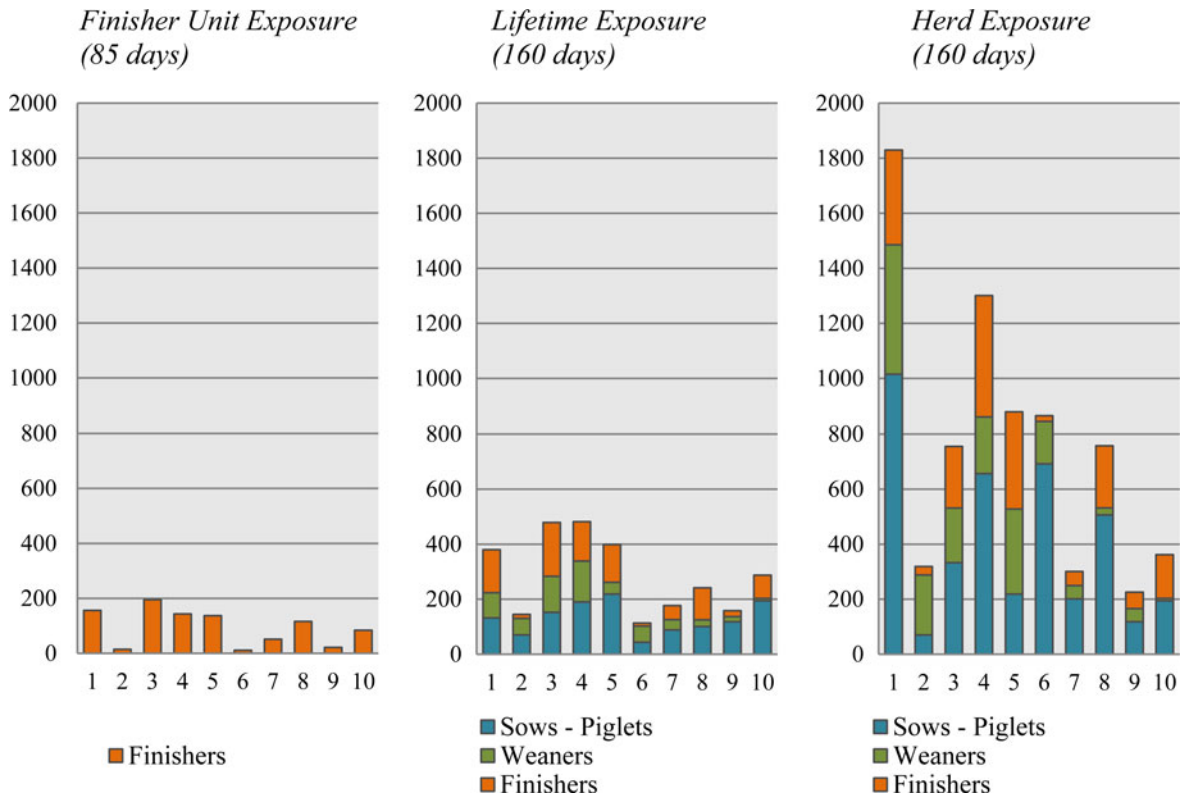


Fig. 3. *Finisher Unit Exposure*, *Lifetime Exposure* and *Herd Exposure*. The total AM use and the distribution between sow piglets, weaners and finishers within the 10 finisher batches. Numbers 1–5 denote the initially high users and 6–10 depict the initially low users.

use on the average proportion of tetracycline-resistant aerobic bacteria (Fig. 5).

No significant effect was observed for *Finisher Unit Exposure* or *Herd Exposure* (result not shown).

Association between AM use and metagenomic measured resistance

No significant effect was observed of *Finisher Unit Exposure* for any of the AM classes on their respective AMR gene classes (result not shown).

Figure 6 shows the regression models with 95% confidence intervals for the quantitative effect of *Lifetime Exposure* for the AM classes: aminoglycoside, lincosamide, macrolide, broad-spectrum penicillin, sulfonamide and tetracycline on the AMR gene classes: aminoglycoside, lincosamide, macrolide, β-lactam, sulfonamide and tetracycline. The β-values, P-values and R^2 values for the regression analyses are shown in the same figure. The regression analyses revealed a significant effect of *Lifetime Exposure* for the AM classes: macrolide, broad-spectrum penicillin, sulfonamide and tetracycline on AMR genes for macrolide, β-lactam, sulfonamide and tetracycline, respectively (Fig. 6).

Figure 7 shows the regression models with 95% confidence intervals of the quantitative effect for *Herd Exposure* for the AM classes: aminoglycoside, lincosamide, macrolide, broad-spectrum penicillin, sulfonamide and tetracycline on the AMR gene classes: aminoglycoside, lincosamide, macrolide, β-lactam, sulfonamide and tetracycline. The β-values, P-values and R^2 values for the regression analyses are shown in the same figure. The regression analyses revealed a significant effect of *Herd Exposure* for the AM classes: aminoglycoside, lincosamide and tetracycline on the AMR genes for aminoglycoside, lincosamide and tetracycline, respectively (Fig. 7).

The regression models were re-analysed, excluding the most extreme data point of each set, to assess the robustness of the significant results. The significant effect of *Lifetime Exposure* for the AM classes: macrolide, sulfonamide and tetracycline on the occurrence of macrolide, sulfonamide and tetracycline resistance genes, respectively, remained under the 5% significance level. This was not the case for the significant

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

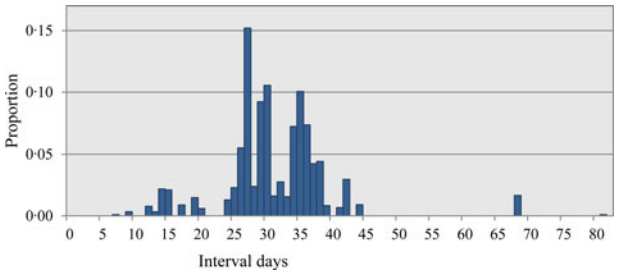


Fig. 4. The distribution of prescription interval days – days between two prescriptions.

effect of broad-spectrum penicillin usage on the occurrence of β-lactam resistance genes (result not shown). Only the effect of *Herd Exposure* for tetracycline on tetracycline-resistant genes remained under the 5% significance level (result not shown). The coefficient of determination (R^2) analyses of the models with significant results revealed that the variation of AMR that could be explained by the *Lifetime Exposure* measurements varied from 0·42 (tetracycline) to 0·72 (sulfonamide) for the variables (Figs 5 and 6), and from 0·47 (aminoglycoside) to 0·67 (lincosamide) for the *Herd Exposure* measurements (Fig. 7).

DISCUSSION

Independent variables

The exposure measurements were derived from different data sources, thereby integrating any errors of the sources in the daily ADDkg.

The length of the rearing period influences the exposure measurements; however, this study did not determine whether the rearing periods should be longer or overlapped in order to assess the effect on the occurrence of AMR genes. Nevertheless, shorter rearing periods reduce the AM amounts, resulting in less variation in the exposure measurements between finisher batches. Longer rearing periods have the opposite effect.

The intervals were calculated based on two assumptions. First, as the intervals were not drug-specific, a prescription of tetracycline followed by a prescription of macrolide counted as an interval because it was assumed that any drug shifts were due to a different disease or a change, as the veterinarian could not observe a sufficient effect caused by the first drug. This is supported by veterinarians' permission to prescribe AMs. Consequently, a new situation is likely to have occurred on the farm if AMs were prescribed with intervals shorter than 35 days. Short intervals result in high daily ADDkg, causing a substantial increase in the measured AM use, as the prescribed amount of AM is divided by fewer days. Second, all prescription intervals of <7 days were assumed to be due to technical issues at the pharmacies, e.g. shortages of a drug or a shift in batches at the pharmacy, or caused by the veterinarian issuing two identical prescriptions (the sub-diagnosis differs) that were handed in on different dates.

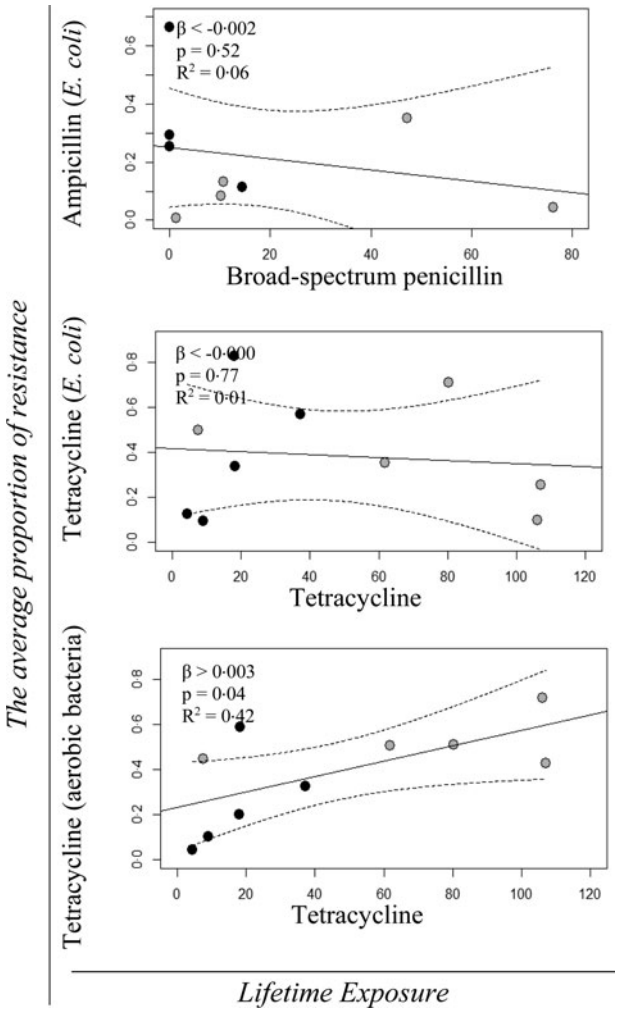


Fig. 5. Univariable linear regression plots (solid line) and 95% confidence interval (dotted lines) of the average resistance proportion of ampicillin and tetracycline resistance from cultivation of *Escherichia coli* on MacConkey agar with and without ampicillin and tetracycline, as a function of *Lifetime Exposure* for the AM classes; broad-spectrum penicillin and tetracycline, respectively, and the average resistance proportion of tetracycline resistance from cultivation of aerobic bacteria on LB agar with and without tetracycline, as a function of *Lifetime Exposure* for the AMc; tetracycline. The grey points denote the initially high users and the black points depict the initially low users. The effect (β), the *P*-value (*P*) and the R^2 value are shown in the top left corner of each model.

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

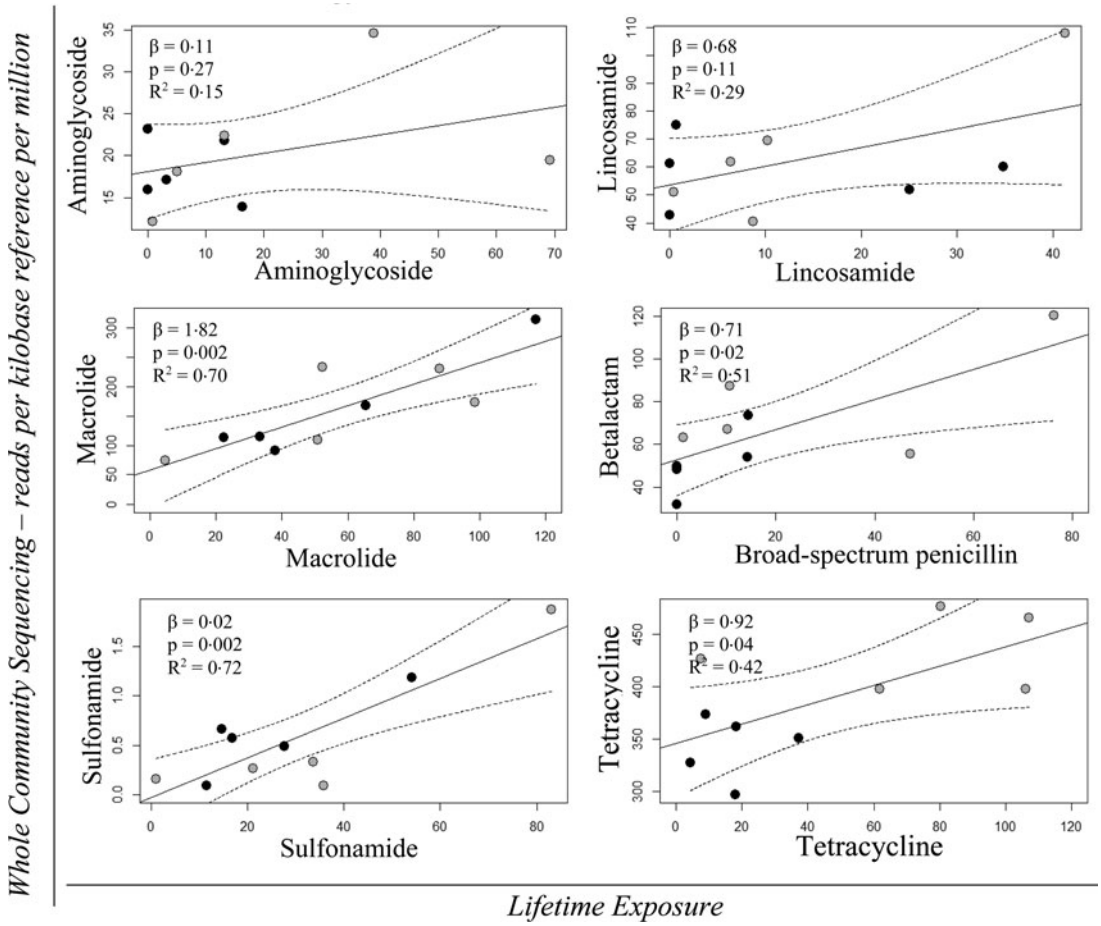


Fig. 6. Univariable linear regression plots (solid line) with 95% confidence interval (dotted lines) of WCS – RPKM of the AMR genes for: aminoglycoside, lincosamide, macrolide, β-lactam, sulfonamide and tetracycline as a function of *Lifetime Exposure* for the AM classes: aminoglycoside, lincosamide, macrolide, broad-spectrum penicillin, sulfonamide and tetracycline, respectively. The grey points denote the initially high users and the black points depict the initially low users. The effect (β), the *P*-value (*P*) and the R^2 value are shown in the top left corner of each model.

Dependent variables

Cultivation using aerobic bacteria and *E. coli*, respectively, are traditional phenotypical methods for assessing the occurrence of AMR in populations of animals [9]. However, the approaches only provide insight into a fraction of the intestinal microbiota, and thus potentially underestimate the actual reservoir of AMR in a population [3, 7, 9].

WCS measures the presence of known resistance genes throughout the microbial community, and therefore does not determine the genetic location of the AMR genes and thereby whether they are likely to transfer from the animal to the human reservoir [3]. A distinction between the two is important since ubiquitous AMR genes may not pose a risk to humans [19]. By contrast, any AMR genes in excessive abundance in habitats with high AM use, such as pig farms, are more likely to be relevant for AMR development [7, 19]. Furthermore, as the ResFinder database contains only AMR genes found in culturable bacteria, a considerable number of ubiquitous functional AMR genes may have been missed [3]. Therefore, although not all AMR genes necessarily constitute a risk to humans, different amounts in faeces from pigs represent an available gene pool from which zoonotic bacteria may obtain resistance genes [19].

Result discussion

The 10 finisher batches varied in rearing pathways (Fig. 1) and the herds in which they were produced differed concerning the number of farms per herd. Thus, on that basis, no pattern or common feature could be observed. Given that the study included

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

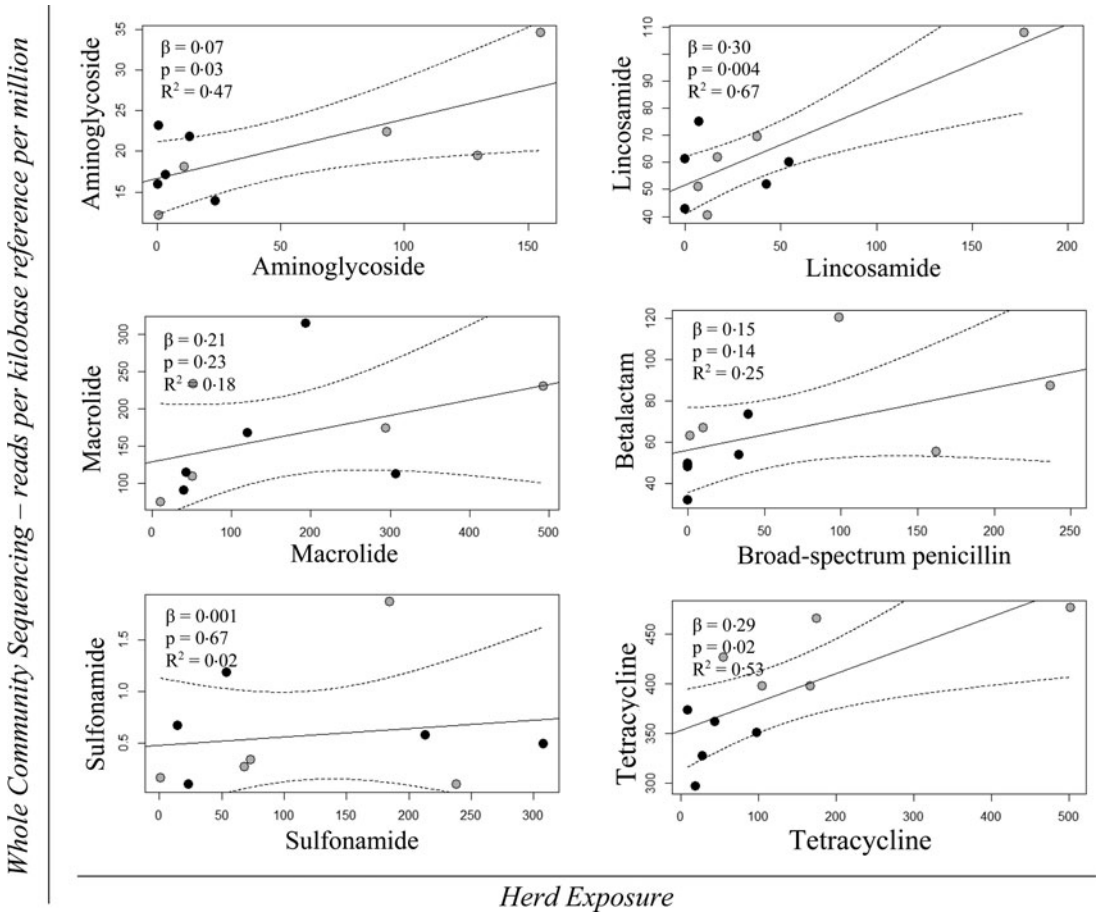


Fig. 7. Univariable linear regression plots (solid plot) with 95% confidence interval (dotted lines) of WCS – RPKM of the AMR genes for: aminoglycoside, lincosamide, macrolide, *β*-lactam, sulfonamide and tetracycline as a function of *Herd Exposure* for the AM classes: aminoglycoside, lincosamide, macrolide, broad-spectrum penicillin, sulfonamide and tetracycline, respectively. The grey points denote the initially high users and the black points depict the initially low users. The effect (*β*), the *P*-value (*P*) and the R^2 value are shown in the top left corner of each model.

only 10 finisher batches, no attempt was made to conduct an analytical adjustment of a potential confounding effect from herd or farm management.

It is notable that the results of the regression models for four out of six combinations of *Lifetime Exposure vs* AMR obtained by WCS (Fig. 6) gave significant results, as did three out of six combinations of *Herd Exposure vs* AMR obtained by WCS (Fig. 7). In order to crudely evaluate the reliability of the results, the individual observations in each of the significant plots that appeared to have the strongest influence on the result were removed and the model was re-analysed. The *Lifetime Exposure* measurement still provided significant results for all but broad-spectrum penicillin, while only *Herd Exposure* remained significant for tetracycline, indicating that the former method of measuring AM use is more sensitive. Furthermore, the measurement of *Herd Exposure* is highly influenced by differences in the units present in each farm, rather than differences in AM use. By comparing the pathways of the finisher batches, large differences can be observed in the number of units per farm, which would greatly affect the measurement (Fig. 1 and Supplementary Fig. S1). The fact that overall the R^2 models of *Lifetime Exposure* explain more of the variation in AMR compared with *Herd Exposure* indicates that *Lifetime Exposure* has the strongest effect on AMR in finisher batches. No effect of *Finisher Unit Exposure* on AMR could be demonstrated; however, use of AM in this unit is limited in terms of both AM classes and amounts [25].

In this study, a significant quantitative association could be demonstrated for *Lifetime Exposure* of tetracycline on AMR across methods of obtaining resistance (Figs 5 and 6), which is in alignment with

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

Munk *et al*., who demonstrated a correlation in AMR between cultivation and metagenomics [3].

The measurements were calculated as total amounts throughout the rearing period for the six specified AM classes, rather than a measurement for each rearing period. Consequently, the measurements do not distinguish between different AM use patterns within the three rearing periods. Batches with the same total AM class usage might relate to use in different units; thus, the rearing period, and the occurrence of AMR for these finisher batches, may not be identical due to rapid change in the occurrence of AMR [13, 36].

In this study, three different approaches for calculating use of AM at finisher batch level were developed. Using an optimal and standardised calculation method is important for setting drug use targets and for direct possible interventions with an expected effect on AMR. *Lifetime Exposure* has the advantage of being independent of production type/rearing site. Furthermore, it follows finisher batches through rearing site(s) in the actual rearing periods, hereby capturing variations in AM use between batches. In contrast to this, the analyses did not consider other factors e.g. duration of treatment or dispensing type, which are known to have an impact on the development and spread of AMR [16]. Overall, this study showed that the entire rearing period should be taken into account when studying the association between AMR and AM use, and revealed that the method developed for calculating *Lifetime Exposure* is an efficient measurement of the effect of AM use on AMR found in finisher batches.

By using metagenomics, we measured the relative abundance of specific genes in the faeces. Even if more DNA fragments were sequenced per sample, there is a likelihood that genes with a very low relatively abundance would not be detected. In the estimation of the association between AM and AMR, the abundance of resistance genes was aggregated to phenotypical level. The estimated associations should only be interpreted at this level and not to gene level. Due to the limited size of this study, an estimation of the quantitative association between the AM usage and abundance of the specific genes was not carried out.

Overall, the study has generated quantitative knowledge of how the usage of AM through the entire rearing period affects the occurrence of AMR in animals at the end of the production. This knowledge will be valuable when assessing effects of alternative AM usage on the occurrence of AMR in the pig production. The validity of these assessments will improve the robustness and precision of the decisions about interventions targeted against reducing the occurrence of AMR in pigs.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0950268817001285.

ACKNOWLEDGMENTS

The authors thank all the farmers for participating in this study.

FUNDING

This work was supported by the Danish Ministry of Food, Agriculture and Fisheries.

CONFLICTS OF INTEREST

None.

REFERENCES

1 **WHO.** The evolving threat of antimicrobial resistance – options for action. World Health Organization, 2012. http://www.who.int/patientsafety/implementation/amr/publication/en/index.html (accessed 11 January 2017).

2 **ECDC/EMEA.** Joint Technical Report: the bacterial challenge: time to react, 2009. http://ecdc.europa.eu/en/publications/_layouts/forms/Publication_DispForm.aspx?ID=199&List=4f55ad51-4aed-4d32-b960-af70113dbb90 (accessed 11 January 2017).

3 **Munk P, *et al.*** A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds. *Journal of Antimicrobial Chemotherapy* 2017; **72**(2): 385–392. doi: 10.1093/jac/dkw415.

4 **Swann MM, *et al.*** *Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine (Swann Report)*. London: H.M.S.O, 1969.

5 **Martel J-L, *et al.*** New trends in regulatory rules and surveillance of antimicrobial resistance in bacteria of animal origin. *Veterinary Research* 2001; **32**: 381–392. doi: 10.1051/vetres:2001131.

6 **Catry B, *et al.*** Antimicrobial resistance in livestock. *Journal of Veterinary Pharmacology and Therapeutics* 2003; **26**: 81–93. doi: 10.1046/j.1365-2885.2003.00463.x.

7 **Marshall BM, Levy SB.** Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews* 2011; **24**: 718–733.

8 **Hu Y, *et al.*** The bacterial mobile resistome transfer network connecting the animal and human microbiomes. *Applied and Environmental Microbiology* 2016; **82**: 6672–6681. doi: 10.1128/AEM.01802-16.

Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

9 **Schmidt GV, *et al.*** Sampling and pooling methods for capturing herd level antibiotic resistance in swine feces using qPCR and CFU approaches. *PLoS ONE* 2015; **10**: e0131672. doi: 10.1371/journal.pone.0131672.

10 **Hammerum AM, *et al.*** Characterization of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins. *Journal of Antimicrobial Chemotherapy* 2014; **69**: 2650–2657. doi: 10.1093/jac/dku180 [doi].

11 **Aarestrup FM.** Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic and Clinical Pharmacology and Toxicology* 2005; **96**: 271–281.

12 **Jensen VF, *et al.*** Correlation between apramycin and gentamicin use in pigs and an increasing reservoir of gentamicin-resistant *Escherichia coli*. *Journal of Antimicrobial Chemotherapy* 2006; **58**: 101–107.

13 **Cavaco LM, *et al.*** Selection and persistence of CTX-M-producing *Escherichia coli* in the intestinal flora of pigs treated with amoxicillin, ceftiofur, or cefquinome. *Antimicrobial Agents and Chemotherapy* 2008; **52**: 3612–3616.

14 **Vieira AR, *et al.*** Association between tetracycline consumption and tetracycline resistance in *Escherichia coli* from healthy Danish slaughter pigs. *Foodborne Pathogens and Disease* 2009; **6**: 99–109.

15 **Chantziaras I, *et al.*** Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *Journal of Antimicrobial Chemotherapy* 2014; **69**: 827–834.

16 **Colloneau L, *et al.*** Guidance on the selection of appropriate indicators for quantification of antimicrobial usage in humans and animals. *Zoonoses and Public Health* 2017; **64**: 165–174.

17 **Aarestrup FM, *et al.*** Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrobial Agents and Chemotherapy* 2001; **45**: 2054–2059. doi: 10.1128/AAC.45.7.2054-2059.2001 [doi].

18 **Agersø, Y, Aarestrup FM.** Voluntary ban on cephalosporin use in Danish pig production has effectively reduced extended-spectrum cephalosporinase-producing *Escherichia coli* in slaughter pigs. *Journal of Antimicrobial Chemotherapy* 2013; **68**: 20569–20572.

19 **Martinez JL, Coque TM, Baquero F.** What is a resistance gene? Ranking risk in resistomes. *Nature Reviews Microbiology* 2015; **13**: 116–123. doi: 10.1038/nrmicro3399.

20 **Emborg HD, *et al.*** Tetracycline consumption and occurrence of tetracycline resistance in *Salmonella typhimurium* phage types from Danish pigs. *Microbial Drug Resistance* 2007; **13**: 289–294. doi: 10.1089/mdr.2007.746.

21 **Rosengren LB, *et al.*** Associations between feed and water antimicrobial use in farrow-to-finish swine herds and antimicrobial resistance of fecal *Escherichia coli* from grow-finish pigs. *Microbial Drug Resistance* 2007; **13**: 261–270.

22 **Timmerman T, *et al.*** Quantification and evaluation of antimicrobial drug use in group treatments for fattening pigs in Belgium. *Preventive Veterinary Medicine* 2006; **74**: 251–263. doi: 10.1016/j.prevetmed.2005.10.003.

23 **Sjölund M, *et al.*** Quantitative and qualitative antimicrobial usage patterns in farrow-to-finish pig herds in Belgium, France, Germany and Sweden. *Preventive Veterinary Medicine* 2016; **130**: 41–50.

24 **Vigre H, *et al.*** Use of register data to assess the association between use of antimicrobials and outbreak of postweaning multisystemic wasting syndrome (PMWS) in Danish pig herds. *Preventive Veterinary Medicine* 2010; **93**: 98–109.

25 **DANMAP.** Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. 2015. http://www.danmap.org/Downloads/Reports.aspx (accessed 11 January 2017).

26 **SEGES.** ANNUAL REPORT 2015. 2016. http://eng.vsp.lf.dk/~/media/Files/PDF - Aarsberetning VSP English/VSPårsberetningUK2015.pdf (accessed 11 January 2017).

27 **Holman DB, Chénier MR.** Impact of subtherapeutic administration of tylosin and chlortetracycline on antimicrobial resistance in farrow-to-finish swine. *FEMS Microbiology Ecology* 2013; **85**: 1–13. doi:10.1111/1574-6941.12093.

28 **Houe H, Gardner IA, Nielsen LR.** Use of information on disease diagnoses from databases for animal health economic, welfare and food safety purposes: strengths and limitations of recordings. *Acta Veterinaria Scandinavica* 2011; **53**: S7.

29 **Stege H, *et al.*** VETSTAT – the Danish system for surveillance of the veterinary use of drugs for production animals. *Preventive Veterinary Medicine* 2003; **57**: 105–115.

30 **Jensen VF, Jacobsen E, Bager F.** Veterinary antimicrobial-usage statistics based on standardized measures of dosage. *Preventive Veterinary Medicine* 2004; **64**: 201–215. doi: 10.1016/j.prevetmed.2004.04.001.

31 **Christiansen MG.** No 1429: The Structural Development in Danish Pig Production 2014 [in Danish only]. SEGES Pig Research Centre. 2014; 11. http://vsp.lf.dk/Publikationer/Kilder/Notater/2014/1429.aspx (accessed 11 January 2017).

32 **Jensen T, *et al.*** Assessment of future production systems for pigs [in Danish only. Summary available in English]. Videncenter for Svineproduktion 2011. http://vsp.lf.dk/Publikationer/Kilder/lu_Rapporter/38.aspx (accessed 11 January 2017).

33 **SEGES.** ANNUAL REPORT 2014. 2015. http://eng.vsp.lf.dk/~/media/Files/PDF - Aarsberetning VSP English/Årsberetning 2014_UK.pdf (accessed 11 January 2017).

34 **Maribo H.** The feeding of suckling piglets [in Danish only]. SEGES Pig Research Centre. 2010; 2. http://vsp.lf.dk/Viden/Foder/Tildeling af foder/Fodring af/Fodring af pattegrise.aspx?full=1 (accessed 11 January 2017).

35 **Jessen O.** No 1523: National average pig productivity 2014 [in Danish only]. 2015; 1–16. http://vsp.lf.dk/~/media/Files/PDF - Publikationer/Notater 2015/Notat_1523.pdf (accessed 11 January 2017).

36 **Abatih EN, *et al.*** Impact of antimicrobial usage on the transmission dynamics of antimicrobial resistant bacteria among pigs. *Journal of Theoretical Biology* 2009; **256**: 561–573.

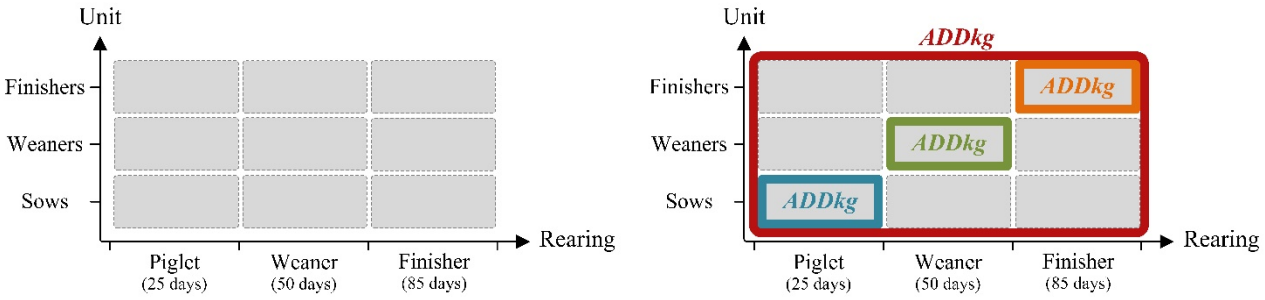
Downloaded from https://www.cambridge.org/core. DTU Library - Tech Info Ctr of Denmark, on 21 Aug 2017 at 12:09:52, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268817001285

3.2 Supplementary material

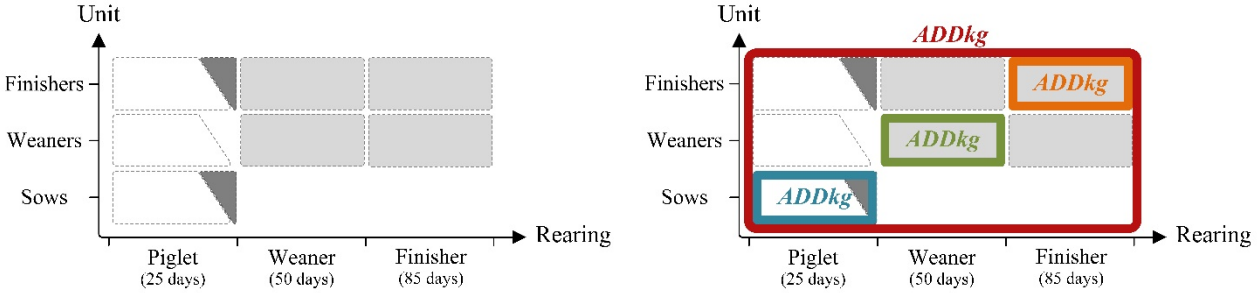
Table 3.2.1. The independent and dependent variables - mean, median and range.

Variables	Mean	Median	Range
Independent			
Aminoglycosides			
Finisher Unit Exposure	0.0	0.0	0.0 - 0.0
Lifetime Exposure	16.0	9.1	0.0 - 69,3
Herd Exposure	43.1	12.2	0.5 - 155.2
Lincosamides			
Finisher Unit Exposure	11.3	3.8	0.0 - 79.4
Lifetime Exposure	12.9	7.6	0.0 - 41.3
Herd Exposure	35.5	14.4	0.0 - 177.2
Macrolides			
Finisher Unit Exposure	27.2	0.0	0.0 - 98.9
Lifetime Exposure	57.0	51.6	4.7 - 117.0
Herd Exposure	160.7	86.5	10.9 - 493.4
Broad-spectrum penicillins			
Finisher Unit Exposure	9.5	0.0	0.0 - 57.1
Lifetime Exposure	17.4	10.4	0.0 - 76.2
Herd Exposure	58.2	21.8	0.0 - 236.5
Sulfonamides			
Finisher Unit Exposure	0.0	0.0	0.0 - 0.0
Lifetime Exposure	29.9	24.3	1.0 - 83.1
Herd Exposure	117.8	70.5	1.0 - 308.2
Tetracyclines			
Finisher Unit Exposure	2.8	0.9	0.0 - 17.2
Lifetime Exposure	45.0	27.8	4.4 - 106.9
Herd Exposure	120.5	76.9	9.33 - 502.1
Dependent			
Cultivation			
MacConkey: Ampicillin	0.2	0.1	0.0 - 0.7
MacConkey: Tetracycline	0.4	0.3	0.1 - 0.8
LB: Tetracycline	0.4	0.4	0.1 - 0.7
Whole community sequencing			
Aminoglycoside	19.8	18.8	12.1 - 34.6
Lincosamide	62.1	60.7	40.4 - 107.8
Macrolide	162.0	140.9	74.7 - 313.7
Beta-lactam	64.9	59.3	31.7 - 120.3
Sulfonamide	0.6	0.4	0.1 - 1.9
Tetracycline	387.3	385.6	296.9 - 475.8

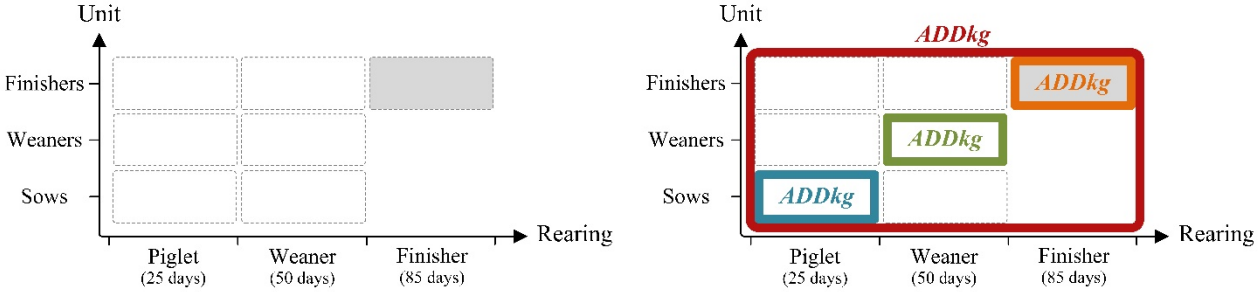
Integrated – no suppliers (ID 1)



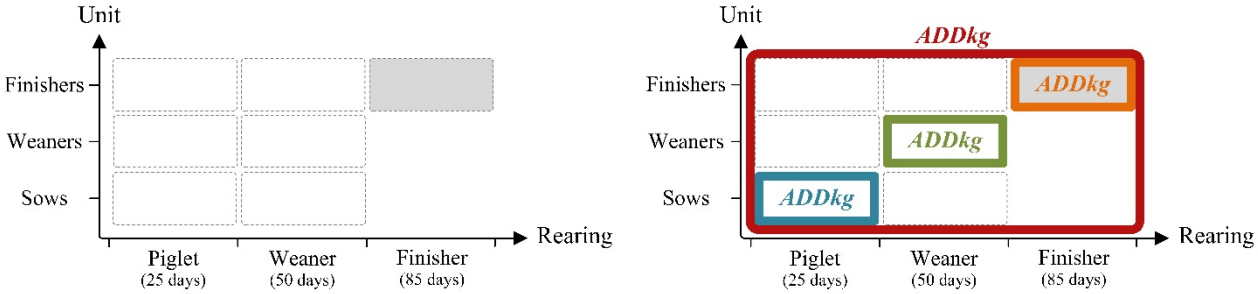
Multisite – 2 suppliers (ID 2)



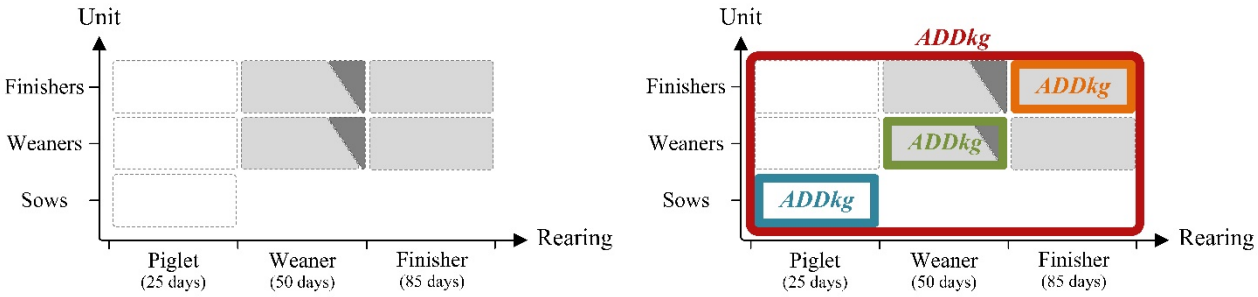
Multisite – 1 supplier (ID 3)



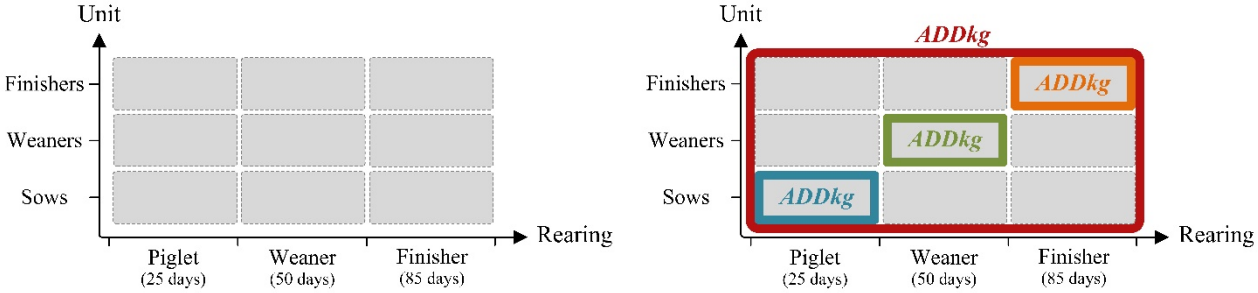
Multisite – 1 supplier (ID 4)



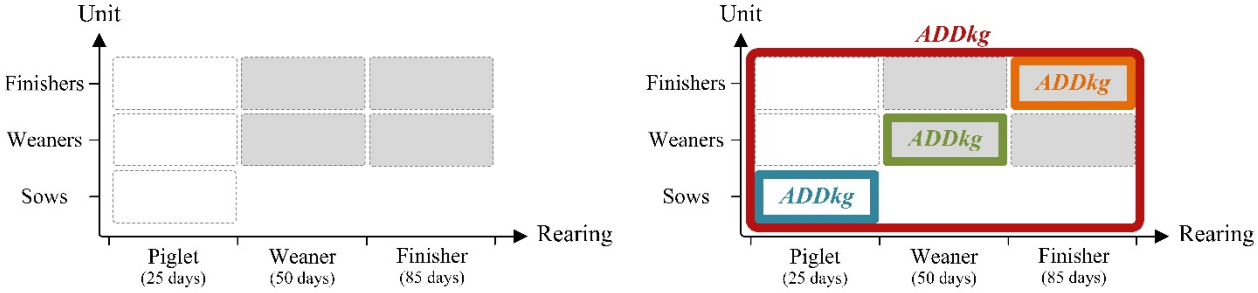
Multisite – 2 suppliers (ID 5)



Integrated – no suppliers (ID6)

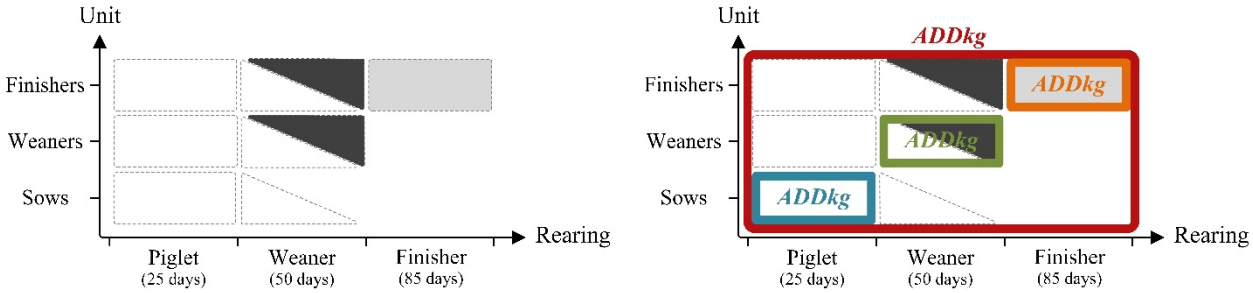


Multisite – 1 supplier (ID 7)



Multisite – 2 suppliers (ID 8)

ID8



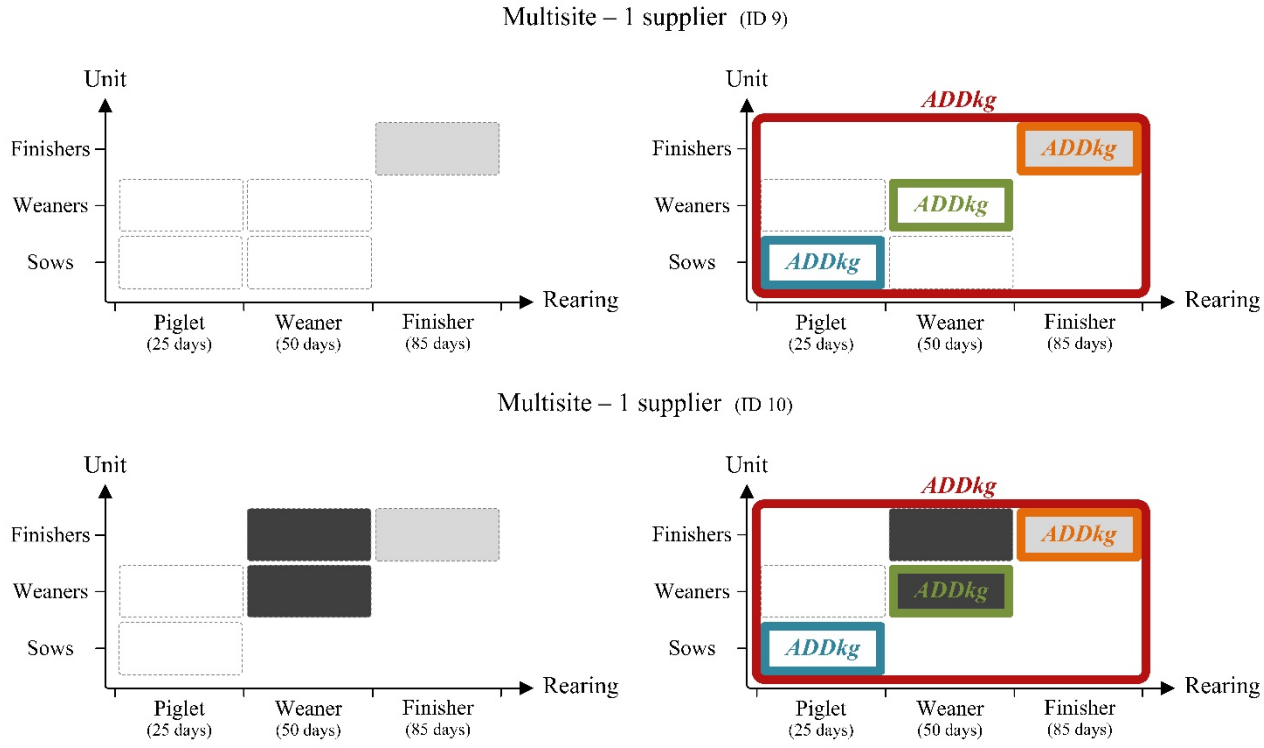


Fig. 3.2.2. The ten finisher batches rearing pathways. A colour shift in a bar denotes that a farm has a different geographical location to the farm where sampling took place. The top graph per ID shows the units within each farm included in the rearing path, and the graph below shows how the exposure measurements of AMU were obtained. The orange square applies to *Finisher Unit Exposure* and therefore comprises the AMU in the finisher-rearing period in the finisher unit. *Lifetime Exposure* applies to the orange, green and blue squares, respectively, and therefore comprises the AM usage in the piglet-rearing period in the sow unit, the weaning-rearing period in the weaning unit and the finisher-rearing period in the finisher unit. The red square applies to *Herd Exposure* and therefore comprises AMU throughout the entire rearing period in all units.

3.3 Discussion

The first objective of the thesis was to develop a method to calculate the AMU in finishers close to slaughter based solely on register-data. Several Danish studies have used methods that either focused on AMU at farm level or farm unit level (Jensen *et al.*, 2006; Emborg *et al.*, 2007; Vieira *et al.*, 2009; Vigre *et al.*, 2010), which may over- and underestimate the amount of AMs a pig has been exposed to during the entire rearing period. In addition, studies have demonstrated that AMU during the earlier rearing stages influences the occurrence of resistance at later rearing stages (Dawson *et al.*, 1984; Dunlop *et al.*, 1998; Rosengren *et al.*, 2007). Subsequently, the developed method aimed at encompassing all rearing stages as primary sources for the selection of AMR.

The pig industry's production statistics provided the average rearing period in days per unit (piglets, weaners and finishers), however, these numbers should only be viewed as indicative (SEGES, 2017). In 2014, the average piglet-nursing period of the highest and lowest preforming farms differed by 2.7 days. While weaners' and finishers' daily weight gain of the highest and lowest performing farms differed by 57g and 120g, respectively (Jessen, 2016). Furthermore, several issues, e.g. aggregation of litters at weaning, uneven growth of pigs, limited space, are often solved by mixing pigs of similar size or by gathering the smaller (uneven) pigs in separate pens, though the procedures cause lack of consistency of individuals in a batch. Therefore, batches of finishers seldom consist of the same individuals throughout the rearing period due to different growth of pigs, mixing of pigs and movements between units during rearing. For finisher farms, farmers also have to take the slaughter house weight limits into account, in order to receive full settlement price for the delivered pigs (Danish Crown, 2017). Therefore, a small amount of pigs are often withhold until they have obtained the appropriate weight. The post-treatment retention period for AMs will also delay the delivering of finishers for slaughter. Consequently, the "finisher batch" represents only a mean rearing path.

The CHR and PDM were used to trace finisher batches back to farm(s) of weaning and birth (Fig. 3.2.2). Due to the ownership status throughout rearing pathways of the finisher batches in this study, the pathways were relatively simple. More complex movement patterns comprising several farms have been found in other studies (Birkegård *et al.*, 2017a). The overall movements of pigs in Denmark is substantial. A study that included movements between farms in Denmark, showed that of the 3086 finisher batches included, 80% were moved at least once (Birkegård *et al.*, 2017a). The many movements are a result of the increased specialisation in production, i.e. breeding animals, 7kg piglets, 30kg weaners, finishers only etc. Nevertheless, difficulties in tracing pigs arise when they are moved between farms. It is mandatory to report data in PMD twice for each movement, first by the farm sending pigs and secondly by the farm receiving pigs (Executive order 598/2017). In this study, a few farmers owing several farms did not register any movements, or their registration only included one of the two required. A previous study found that finisher farms had no movements into the farm in 61% of cases. For the breeder and rearing farms, the numbers were 74% and 51%, respectively (Bigras-Poulin *et al.*, 2007). Therefore, for finisher batches in this study, it was assumed that pigs were only moved between farms at ownership level, when movements between farms were untraceable.

VetStat was used to extract data on AMU. Using the Vigre method to calculate daily usage within farm units, provided the foundation for the approaches assessing relevant exposure with an effect on the occurrence of AMR in finisher batches close to slaughter (Vigre *et al.*, 2010). Based on the rearing pathway, daily AMU amounts were summed at different levels; finisher unit exposure, lifetime exposure and herd exposure. The usage was estimated as the number of kilogram pigs that could be treated during a specified rearing period. The advantage of the measurement was that it could be summed irrespectively of the unit (piglets/sows, weaners and finishers). On the negative side, it was very hard to comprehend. The AMU measurements could beneficially have been converted into Treatment Incidence of the rearing period, by including kg animals at risk (sow - 200kg, weaner - 15kg, finisher - 50kg), which would provide the number of treatment days in the batches' rearing period (Jensen, Jacobsen and Bager, 2004; Callens *et al.*, 2012; Postma *et al.*, 2016, 2016a). Changing the unit of lifetime AMU to treatment days revealed that the R-squared (R^2) of sulfonamides worsened, the R^2 of aminoglycosides and extended-spectrum penicillins remained the same, and the R^2 of lincosamides, macrolides and tetracycline improved. Therefore, using the sow weight had a negative effect on the sulfonamides primarily used in the sows-piglets unit, however, due to the weight of weaners and finishers, a beneficial effect on AMs primarily used in the weaner and finisher unit was found (Appendix A, Figure A1). Because the AMU in piglets cannot be established based on VetStat data, the biomass adjustment had not yet been considered in the lifetime AMU calculations. For the same reason, it was decided to proceed with the measuring unit; ADDkg/pig.

The three approaches have the advantage of being independent of production-system/rearing site, as the method follows finisher batches through rearing site(s) in the actual rearing periods, hereby capturing variations in AMU between batches. However, none of the preliminary analyses included other risk factors, e.g. duration of treatment or dispensing-type, which are known to affect the emergence and spread of AMR (Zhang *et al.*, 2013; Collineau *et al.*, 2017).

The AMR resistance was obtained by cultivation using aerobic bacteria and *E. coli*. These are traditional phenotypical methods when assessing the occurrence of AMR in populations of animals (Turnidge and Paterson, 2007; Schmidt *et al.*, 2015), e.g. DANMAP. In particular, *E. coli* are often used for epidemiological studies of AMR in the food chain due to their ubiquitous occurrence, their tendency to easily develop AMR, their ability to transfer resistance genes and their potential to work as an AMR source (Turnidge and Christiansen, 2005). However, the cultivation methods only provide insight into a fraction of the intestinal microbiota of which the majority are anaerobic, but they are all affected by AMU (Dawson *et al.*, 1984; Holman and Chénier, 2015). Consequently, the methods potentially underestimate the actual reservoir of AMR in the microbiota of pigs (Marshall and Levy, 2011; Schmidt *et al.*, 2015; Munk *et al.*, 2017).

The shotgun metagenomic sequencing (called WCS in manuscript I and II) measures the relative presence of resistance genes throughout the microbial community. The method does not determine whether the genetic location of the AMR genes is intrinsic or acquired, thus, the method cannot establish if the genes are likely to be transferred from the pig to the human reservoir (Munk *et al.*,

2017). A distinction between the two is important since intrinsic AMR genes may not pose a risk to humans (Martinez, Coque and Baquero, 2015). However, AMR genes in excessive abundance in habitats with high selection pressure from AMU, such as pig farms, are more inclined to be the result of acquired AMR, therefore, more relevant for the emergence and spread of AMR in and between reservoirs (Marshall and Levy, 2011; Martinez, Coque and Baquero, 2015). The ResFinder database contains only AMR genes found in culturable bacteria, which in the gut microbiota of pigs could be as little as 1% (Holman and Chénier, 2015; Munk *et al.*, 2017). Therefore, a considerable number of ubiquitous intrinsic AMR genes may have been missed (Munk *et al.*, 2017).

For tetracycline, significant quantitative associations could be demonstrated for lifetime AMU on AMR obtained by cultivation (anaerobe bacteria) and shotgun metagenomics sequencing, which was in alignment with a previous study that demonstrated a correlation in AMR between cultivation and metagenomics (Munk *et al.*, 2017). It is worth noticing that the *E. coli* cultivation did not provide any significant AMU and AMR associations across methods of measuring AMU, which was unexpected given its widespread usage as indicator-bacteria for resistance. Whether the cultivation method was biasing the resistance outcome, or the level of resistance found was a correct measure of phenotypic resistance could not be determined. However, resistance in *E. coli* might be affected by several other factors than the tetracycline lifetime usage alone. This is an important predicament to solve in order to being able to describe the link between phenotypic resistance and resistance gene abundance.

Of the three approaches for measuring AMU assessed, the finisher exposure was the poorest, which indicates that the occurrence of AMR in finisher batches close to slaughter is not solely affected by the AMU in the finisher unit. This is in alignment with previous studies, which have found that off-spring are affected by the sow's previous tetracycline usage (Mathew *et al.*, 2005), and that the AMR genes persist from sows through rearing (Birkegård *et al.*, 2018). Furthermore, thefarm of origin affects the spread of AMR in pigs at the subsequent farm (Dawson *et al.*, 1984). A crude evaluation of the reliability of the significant results, by removing the observation in each of the significant plots that appeared to have the strongest influence on the result, demonstrated that the lifetime exposure provided most significant results. In contrast, the herd exposure remained only significant for one AM, indicating that the former method of measuring AMU is more sensitive. In addition, the measurement of herd exposure is highly influenced by differences in the units present in each farm, rather than the differences in AMU. By comparing the pathways of the finisher batches, large differences can be observed in the number of units per farm, which would greatly affect the measurement (Fig. 3.2.2.). Overall, the R^2 explained more of the variation in AMR of the lifetime exposure models compared with herd exposure, indicating that the former has the strongest effect on AMR in finisher batches.

The exposure measurements were calculated as total amounts throughout the rearing period for specified AM-classes, rather than a measurement for each rearing period. This evened out the distinction between different AMU patterns within the three rearing periods, e.g. differences in parenteral and peroral dispensing. Therefore, finisher batches with the same AM-class usage might relate to use in different units, and the occurrence of AMR may not be identical for these batches due

to changes in occurrence of AMR over time (Dunlop *et al.*, 1998; Cavaco *et al.*, 2008; Abatih *et al.*, 2009; Collineau *et al.*, 2017). Subsequently, a limitation that might account for some of the non-explained variation observed between batches.

Although the lifetime AMU was found to be a good method for measuring the effect of AMU on AMR, the linear association could be the result of the study objects selected. Of the studies 10 finisher batches, 5 batches came from farms with very high usage and 5 batches came from farms with very low usage, provided two extremes between which to plot. Therefore, the association between AMU and AMR cannot for certain be presumed to be linear, and a random selection of finisher batches might have revealed a different association. The difference between the two high and low groups could be further strengthened by lack of typical risk factors affecting the AMR. First, for each finisher batch, one farmer owned all farms included in a rearing pathway. Therefore, as the vet connected to the farmer had Health Advisory Contracts with all farms owned by the farmer, thus, the farms within a rearing pathway would be expected to have similar treatment strategies, i.e. choice, dose and dosage of AMs, as usage in a farm (Vigre *et al.*, 2010; Hybschmann *et al.*, 2011). Since the occurrence of resistance genes has been demonstrated to be affected by the occurrence in farms of origin (Dawson *et al.*, 1984; Birkegård *et al.*, 2018), the effect of AMU per unit may therefore be reinforced over time. Secondly, the AMR level in the batches did not encounter AMR contaminants from outside-pigs, which could have altered the AMR composition and level (Dawson *et al.*, 1984).

4. Objective II

4.1 Manuscript II

Validation of the register-based lifetime antimicrobial usage measurement for finisher batches based on comparison to recorded antimicrobial usage at farm level

V. D. Andersen*, P. Munk, L. V. de Knegt, M. S. Jensen, F. M. Aarestrup and H. Vigre.

The National Food Institute, Technical University of Denmark, Lyngby, Denmark

*Corresponding author.

Published in Epidemiology and Infection 2018 [Epub ahead of print]
DOI: 10.1017/S0950268818000134

Epidemiology and Infection

cambridge.org/hyg

Original Papers

Cite this article: Andersen VD, Munk P, de Knegt LV, Jensen MS, Aarestrup FM, Vigre H. Validation of the register-based lifetime antimicrobial usage measurement for finisher batches based on comparison with recorded antimicrobial usage at farm level. *Epidemiology and Infection* https://doi.org/10.1017/S0950268818000134

Received: 9 October 2017
Revised: 15 December 2017
Accepted: 8 January 2018

Key words:
Antimicrobial resistance; antimicrobials usage; finishers; validation

Author for correspondence:
Vibe Dalhoff Andersen, E-mail: vdan@food.dtu.dk

© Cambridge University Press 2018

CAMBRIDGE UNIVERSITY PRESS

Validation of the register-based lifetime antimicrobial usage measurement for finisher batches based on comparison with recorded antimicrobial usage at farm level

V. D. Andersen, P. Munk, L. V. de Knegt, M. S. Jensen, F. M. Aarestrup and H. Vigre

National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark

Abstract

Assessing the relationship between antimicrobial usage (AMU) and antimicrobial resistance (AMR) requires the accurate and precise utilisation of register data. Therefore, validation of register-based data is essential for evaluating the quality and, subsequently, the internal validity of studies based on the data.

In this study, different smoothing methods for Veterinary Medicine Statistic Program database (VetStat)-records were validated by comparing these with farm-records. Comparison between measurements included accuracy as; completeness and correctness, and precision as; a relative difference of the error, correlation with Fisher's *z* transformation and reliability coefficient. The most valid methods of those examined were then used in re-analyses of the abundance of AMR genes in 10 finisher batches from a previous study.

Improved accuracy was found when detailed smoothing methods were applied. Although the precision also increased, the effect was not as pronounced, as the usage estimate of all smoothing methods deviated moderately compared with the farm-registrations. Applying the most valid methods to the 10 finisher batches increased estimates of statistical model fit for aminoglycosides, lincosamides, tetracyclines and decreased estimates of statistical model fit for macrolides. The estimates of statistical model fit for sulfonamides and broad-spectrum penicillins remained the same.

Through refined data transformation, VetStat-records can be used to calculate a daily amount of AMU per pig reflecting the true usage accurately and moderately precisely, which is the foundation for calculating lifetime AMU.

Introduction

As the emergence and spread of antimicrobial resistance (AMR) bacteria is increasing worldwide, an understanding of the complex associations between antimicrobial usage (AMU) and AMR is urgently needed [1]. The AMU is generally acknowledged as the main cause. However, less is known of the quantitative relationship between AMU and AMR, as well as the interrelational effects between usage in humans, agriculture and veterinary sectors [2, 3]. Due to the potential risk of conveying resistance from animal microflora to human pathogenic bacteria, AMU for animals has gained increased attention [4].

Since antimicrobials (AMs) are vital for the treatment of bacterial diseases in veterinary medicine, responsible AM interventions aimed at reducing usage must be sufficiently effective to reduce AMR without compromising treatment options and animal welfare. Consequently, knowledge of the quantitative 'AMU-AMR' relationship is fundamental in order to obtain predictable results from interventions targeting AMU in animal production [5].

Several surveillance databases on AMU for animals have been established [6]. Among the first was the Danish Veterinary Medicine Statistic Program database (VetStat), which records purchases of medicines prescribed for animals [7, 8] and is commonly used for epidemiological studies of AMU–AMR relationships in Danish production animals [9–12]. As data from VetStat lack information on actual usage in farms, studies using these data share a mutual challenge in accuracy and precision compared with primary data and should, therefore, be validated [13, 14].

Farmers are obliged to register AMU for production animals on a daily basis. These records are often summed either by the farmer or by the veterinarian for the period between two consecutive visits by the veterinarian, which usually occurs at intervals of 30–65 days depending on production type and Health Advisory Contract. In this study, the farm records were the summed daily AMU between consecutive veterinarian visits. The farm records are not mandatory, but they provide the farmer and veterinarian with a quick overview of AMU and remnants from recent prescriptions. Validation measurements of VetStat-records compared with farm-records should include (1) accuracy, as the completeness and correctness

Downloaded from https://www.cambridge.org/core. IP address: 85.204.133.39, on 04 Mar 2018 at 10:27:15, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268818000134

and (2) precision, as the correlation, as the relative difference and as the coefficient of reliability of VetStat data. These measurements will demonstrate the quality of the data, which will be supportive when evaluating the trustworthiness of studies of AMU–AMR relationships using such data [13–15].

Currently, the most influential exposure characteristics of AMs, e.g. route of administration, level of dose, or duration of treatment, have not been fully determined in relation to the selection of AMR [5]. In previous studies utilising VetStat as the data source, data on AMU for pig herds have been extracted at the unit (piglets-sows/weaners/finishers) or farm level for periods of 6–12 months prior to sampling [10, 12]. This constitutes minimal differentiated estimates that do not take into account the variations within the extracted period in question. A study used a method that summed up a daily AMU as doses for finisher batches from birth to slaughter, calculating the lifetime AMU through the movements between units, thus, the method was independent of rearing site and captured variations over time [9]. In the same study, the daily usages were calculated by smoothing the amount (a recorded entry) based on days between records. Subsequently reflecting the number of days between one record and the next, within each age-group unit per farm. In contrast, this way of smoothing data does not take into account that different AMs and dispensing-types may be used differently by the farmer.

The objective of this study was to validate five different methods to smoothing VetStat data to estimate the number of ADDkg per pig day, reflecting the 'true' usage at the farms by comparing the results to farm-records in terms of accuracy and precision. The results from a previous study focusing on the effect of AM lifetime exposure on the abundance of AMR genes were then re-analysed with the most valid methods of those examined, for calculating AMU at finisher batch level. Two different farm size adjustments were then used to evaluate the same methods.

Materials and methods

Data sources

Two data sources on AMU were applied in this study: farm-records and VetStat-records.

The farm-records were manually registered by the owners or employees and contained information on the amount of an AM product used, including the dispensing-type, within the age-groups; piglets-sows, weaners and finishers, during specified periods. The farm-records were conveniently collected during farm visits related to an ongoing AMU-AMR study consisting of 83 randomly identified farms. A total of 25 farmers were asked to participate and 12 accepted. A total of 745 records on AMU were obtained, comprising 12 farm owners, 16 farms and 23 units within the period from January 2014 to May 2016.

Data from VetStat contains records on purchased medicines prescribed by veterinarians for animals. Each record has information on the product name, active-substance, dispensing-type, amount, target species, age-group, diagnosis group and farm code (ID) [7]. Data from VetStat were extracted from 1 year before the first farm recorded date to 3 months after the last of each farm to establish sufficient buffer time before and after the study periods to account for negative entries [16]. The data were then cleaned according to guidelines by correcting mismatches of animal species and/or age-group through cross-validating the data with Central Husbandry Register (CHR) data [16].

In order to produce comparable data across records, active compounds were converted into a unit measuring how many kilograms of pig could be treated per day, known as – Animal Defined Daily Doses per kilogram (*ADDkg*) [17].

Two sources of biomass estimates were applied as the adjustment factor for farm size; (i) number of pigs on any given day at the farms, obtained from the CHR, where all farms with production animals are recorded and (ii) the yearly production adjusted to the number of pigs on any given day, obtained from the Pig Movement Database (PMD) [7]. The CHR stores information on a farm code (ID), which refers to a specific geographical location and includes information such as ownership, animal species and the number of animals per age-group (sows/weaners/finishers), on any given day. Although sows and piglets are in the sow unit, the number of sows is included in this age-group, since piglets are not registered in the CHR. In the PMD, the number of pigs, date, ID of origin farm and ID of destination farm for each movement is recorded [7].

Estimation of AMU

Validation

The usage of an AM product (l), during a period (k), in an age-group (j) (piglets-sows/weaners/finishers) in a farm (i) was estimated as $Doses_{i,j,k,l}$ with the unit; *ADDkg/pig day*, using formula (1):

$$\#Doses_{i,j,k,l}[ADDkg/pig\ day] = \frac{\#mg_{i,j,k,l}}{\#days_k * ADDkg_l * \#pigs_{i,j,k}}$$

where: *#mg* = the amount of an AM product registered as usage or recorded as a purchase in a specific farm/age-group/period, *#days* = the number of days of the period when the recorded amount was used, *#pigs* = the number of sows/weaners/finishers on any given day registered in CHR, or the yearly production adjusted to the number of pigs on any given day registered in PMD.

The *#days* was calculated using five different methods. The first method (1) assumed that the AMU in a farm recorded period was equivalent to the purchases of AMs in that recorded period. The other four methods (2–5) were all calculated assuming that the amounts of recorded AM products were used in a period between one recorded date and the next. The subsequent date was defined based on different assumptions related to usage pattern over time at the farms. Consequently, the four smoothing methods differed in the number of days (*#days*) between one record entry date and the next, when the age-group, dispensing-type and antimicrobial class (AMC) alternately and together were taken into account (Fig. 1). In the less detailed method 2, the *#days* between two record entries was set at the age-group level, assuming that a new record of any AM product was due to the previous recorded AM products were consumed by that age-group. Method 3 assumes that when a new record of an AM product of either parenteral or peroral dispensing occurs within an age-group, all the former AM products of the same dispensing-type were consumed. Method 4 assumes that when new recorded AM product of an AMC occurs within an age-group, all the former recorded AM products of the same AMC, irrespectively of dispensing-type, were consumed. Method 5 was a combination of methods 3 and 4 (Fig. 1).

The calculation of *#days* was based on three assumptions. First, if the *#days* was less than 8 days, the following subsequent record

Downloaded from https://www.cambridge.org/core. IP address: 85.204.133.39, on 04 Mar 2018 at 10:27:15, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268818000134

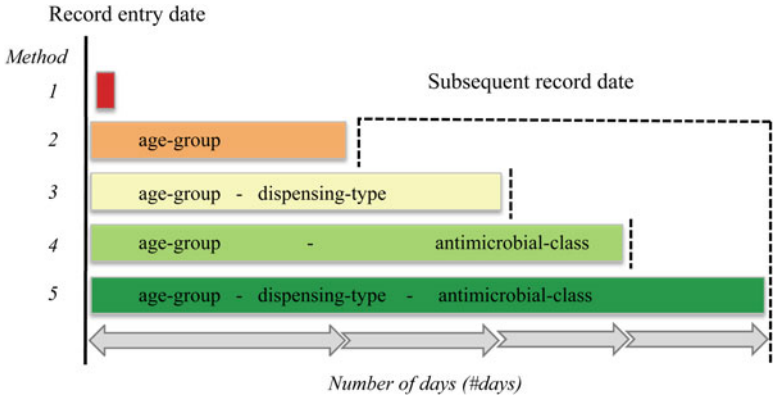


Fig. 1. Illustration of the differences in *Number of days (#days)* for the five methods of calculating antimicrobial usage at the farm level. For method 1, the *#days* was based on the farm record periods. From methods 2 to 5, the *#days* increased as the intervals between one record entry date and the next increased when similar records based on age-group, dispensing-type and antimicrobial-class (AMC) were matched.

date was used instead. Second, if no subsequent date was found, the mean of the former was applied. If no subsequent date was found and no mean of prior *#days* was available, 365 days was utilised. Third, all *#days* exceeding 365 days were substituted by 365 days.

The calculated $Doses_{i,j,k,l}$ obtained for methods 2–5 were date-specific estimates. In order to compare these with the farm-records, the date-specific estimates were summed equivalent to the periods during which the farm-records were calculated and a daily average $Doses_{i,j,k,l}$ usage was calculated.

Re-analyses

For the ten finisher batches from the study [9], the date-specific lifetime AMU (Doses) in the different age-groups was re-calculated by means of formula 1 for the AMCs; aminoglycosides, broad-spectrum penicillins, lincosamides, macrolides, sulfonamides and tetracyclines, using the most valid methods. Furthermore, two different biomass adjustments were applied as the number of pigs on any given day; (A) the CHR and (B) the PMD.

The number of Doses was summarised at AMC level for each rearing period per unit, based on the finisher batches' rearing periods in days; days 1–85 in the finisher unit, days 86–135 in the weaning unit and days 136–160 in the sow unit [18], where day 1 corresponds to the day of sampling. The number was then adjusted to suit the proportion of animals being moved from a farm. Subsequently, for each AMC, the lifetime AMU were calculated for each finisher batch by summarising Doses through the rearing pathways. Even though AMU for sows was included in the usage for piglets, previous studies have shown that this affects the abundance of AMR genes in the piglets' microbiota, thus, it was assumed equivalent to usage for piglets [19].

Data analyses

Validation

Throughout the validation, the VetStat estimates were compared against the farm-record estimates, which were assumed to be the 'true' state of AMU at the farms.

For the accuracy and precision assessments of the relationship between farm-records and VetStat-records, the calculations performed for the observations were mutually independent and dependent, respectively. Consequently, to adjust for potential within-level clustering, all of the validation results were average-adjusted by farm, age-group, dispensing-type or AMC levels to assess the impact of clustering compared with the crude estimates.

Accuracy – completeness and correctness

The completeness constitutes the observed number of VetStat-records compared with the number of farm-records ($a/(a+c)$) and the correctness constitutes the number of correctly identified VetStat-records compared with the number of VetStat-records that were found ($a/(a+b)$), set in a 2×2 table [13, 14].

Precision – relative difference

The relative difference of the error was calculated as the absolute difference between farm and method, divided by the arithmetic mean of the usage given by farm and method ($rd_{\text{error}} = (Doses_{\text{farm}} - Doses_{\text{method}})/((Doses_{\text{farm}} + Doses_{\text{method}})/2)$).

Precision – correlation coefficient

The correlation coefficient ($\boldsymbol{r}_z$) was calculated by applying Fisher's z transformation ($\boldsymbol{r}_z = (e^{2z}-1)/(e^{2z}+1)$, where $z = 0.5ln((1+\boldsymbol{r})/(1-\boldsymbol{r}))$ [20]. The adjusted $\boldsymbol{r}_z$ should be interpreted as the general correlations between farm and method at the level of adjustment. The averaged correlations are less affected by sampling distribution skew, suggesting a less biased statistic [20].

Precision – reproducibility (reliability coefficient)

The reliability coefficient ($\rho_{xx} = 1/(1 + (\sigma_{\text{Error}}/\sigma_{\text{Dosesfarm}})^2)$, where $Error = Doses_{\text{farm}} - Doses_{\text{method}}$), between the $Doses_{\text{farm}}$ and $Error$ obtained, was calculated for each of the five methods [21]. The reliability coefficient describes the average magnitude of the error, the reproducibility. For linear regression, this equals the bias factor; $\beta_{\text{observed}} = \rho_{xx} * \beta_{\text{true}}$ and thus, can potentially be used for adjustment of β_{observed} [21]. Subsequently, the effect estimates obtained in the re-analysed linear regression models presented below were adjusted for the attenuation effect of data error.

Re-analyses

To investigate the influence of the validation results of this study, the findings from the previous study [9] of the effect of six AMCs on the abundance of the same classes of AMR genes were re-analysed by applying the most valid methods in calculating the lifetime AMU. In that study, AMR genes for the classes: aminoglycoside, lincosamide, macrolide, beta-lactam, sulfonamide and tetracycline were obtained using whole community sequencing (WCS) and were measured as reads per kilobase reference per million [22].

Downloaded from https://www.cambridge.org/core. IP address: 85.204.133.39, on 04 Mar 2018 at 10:27:15, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268818000134

The lifetime AMU measure, CHR adjusted (A), for the ten finisher batches was used in linear regression re-analyses to assess each effect on the abundance of AMR genes by evaluating the changes in adjusted R-squared (Adj.R^2), Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). In addition, the reliability coefficient of the most valid method of the presented was applied to adjust the β-coefficients from the linear regression re-analyses.

Finally, the difference of effect of the two lifetime AMU measures, (A) CHR adjusted and (B) PMD adjusted, was evaluated.

Tools

WPS Workbench, Version: 3.1.1.0.0, Microsoft Excel 2016 and R, version 3.3.3 were used for data processing and data analyses.

Results

While cleaning the VetStat data, 19 records were encountered that could not be corrected. Some AM products were prescribed and purchased (recorded in VetStat) one time only, but the usage of these could not be found in the farm-records. In addition, AM products were recorded for one age-group but registered as usage at the farm for another age-group, or for two age-groups.

Validation

Completeness and correctness

Table 1 shows the completeness and correctness results obtained by comparing $Doses_{farm}$ to the five $Doses_{method}$, respectively. The smoothing methods from 1 to 5 had a positive effect on the completeness, which increased from 0.60 to 0.86 and a minor negative effect on the correctness, which decreased from 0.91 to 0.84 (Table 1). The results obtained when performing the average adjustments at farm, age-group, dispensing-type and AMC levels led to a decrease in the overall completeness and correctness results, though the beneficial trend when smoothing remained the same (Table 1).

Table 1. The correctness and completeness of $Doses_{method}$ 1 to 5, compared with $Doses_{farm}$ at population level and average-adjusted at farm, age-group, dispensing-type and antimicrobial-class levels

Method	1	2	3	4	5
Completeness					
Study population	0.60	0.72	0.75	0.83	0.86
Adjusted by					
Farm	0.56	0.69	0.74	0.80	0.83
Age-group	0.59	0.71	0.75	0.83	0.86
Dispensing-type	0.60	0.74	0.78	0.85	0.87
Antimicrobial-class	0.62	0.73	0.76	0.82	0.83
Correctness					
Study population	0.91	0.89	0.87	0.85	0.85
Adjusted by					
Farm	0.88	0.85	0.83	0.82	0.82
Age-group	0.91	0.88	0.87	0.85	0.85
Dispensing-type	0.89	0.86	0.85	0.82	0.82
Antimicrobial-class	0.91	0.88	0.86	0.84	0.84

Relative difference

The distributions of the rd_{error} for the smoothing methods are shown in Figure 2, illustrating that the number of farm-records not found by the smoothing method ($rd_{error} = 2$) decreased from $Doses_{method}$ 1 to 5. However, concurrently, the number of spurious records ($rd_{error} = -2$) was shown to increase, while the distribution of rd_{error} narrows around zero going from method 1 to 5.

The boxplots of the rd_{error} of the five $Doses_{method}$, compared with $Doses_{farm}$ show that the 0.75 quantile decreases substantially and the rd_{error} observations together with the median move toward zero from method 1 to 5 (Fig. 3). Furthermore, it shows that going from method 1 to 5, the mean and the range of the standard deviation of the rd_{error} decreases towards zero (Fig. 3).

Since the smoothing methods (*#days*) depended on similar VetStat-records regarding the age-group, dispensing-type and AMC levels, the rd_{error} of the five $Doses_{method}$ was average-adjusted accordingly. Boxplots at an age-group level were in concordance with general findings (Fig. 4). In contrast, boxplots at dispensing-type level revealed that method upscaling from 1 to 5 was beneficial for parenteral dispensing, but not for peroral (Fig. 5). For peroral dispensing, method 3 provided a better result for the rd_{error}.

The boxplots of the rd_{error} of the five $Doses_{method}$ at farm level show considerable variation between farms, which is most likely to be related to the difference seen between dispensing-types (result not shown). Similar observations were made at AMC level and at AMC combined with dispensing-type level, (result not shown).

Correlation coefficient

In Table 2, the correlation coefficient ($\boldsymbol{r}$) between the $Doses_{farm}$ and the five $Doses_{method}$, show that by incorporating age-groups, dispensing-type and AMC in the smoothing methods, the correlation also increases. This mainly follows the beneficial trends of smoothing from the completeness and relative difference of the error results.

In relation to the z average-adjusted correlation coefficient (r_z) of the five $Doses_{method}$, the farm-level adjustment changed the results most, followed by age-group, dispensing-type and AMC level. However, the upscaling smoothing method trend remained the same, independent of the average adjustment level (Table 2). Furthermore, regardless of the level at which the average adjustment is performed, the r_z remains within a narrow range.

Reliability coefficient

For the five smoothing methods, the coefficient of reliability (ρ_{xx}) ranged from 0.60 to 0.68 and the average adjustment at farm, age-group, dispensing-type and AMC levels had a similar decreasing effect on the values compared with previous findings. However, the beneficial upscaling method trend remained the same, independent of the average-adjustment level (Table 2). The reliability coefficients of smoothing methods 1 to 5 were all values below 1, meaning that the methods underestimate the AMU compared with the 'true' state, obtained from the farm-records (Table 2).

Downloaded from https://www.cambridge.org/core. IP address: 85.204.133.39, on 04 Mar 2018 at 10:27:15, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268818000134

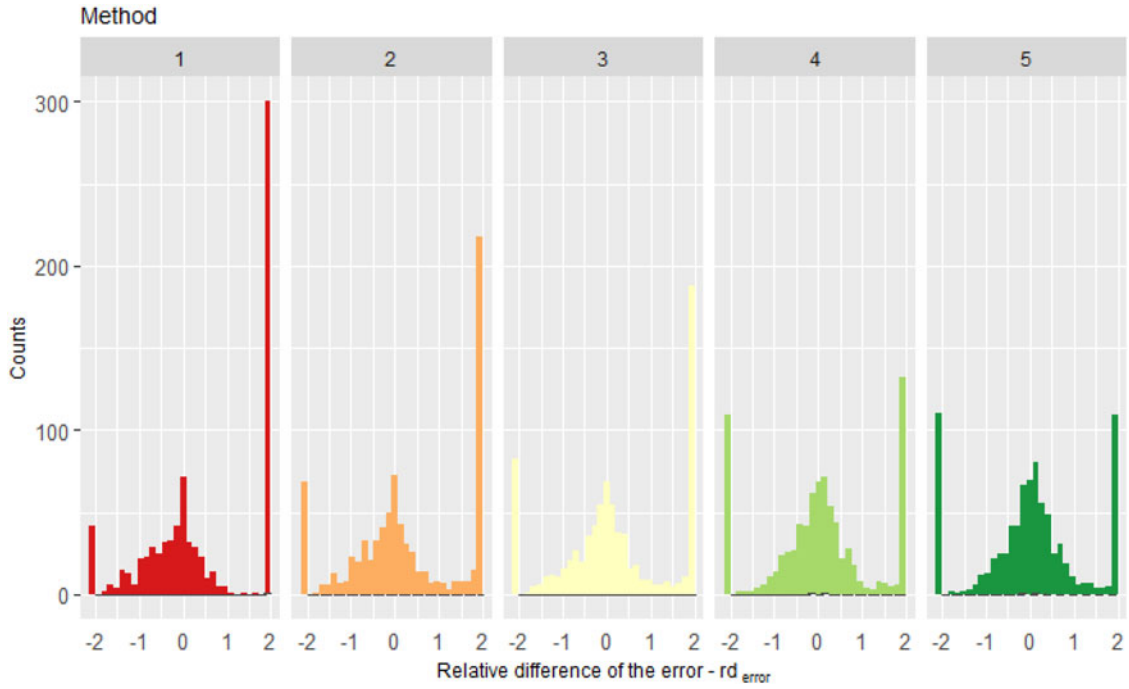


Fig. 2. The count distribution of the relative difference of the error (rd_{error}) for the $Doses_{method}$ 1 to 5 compared with $Doses_{farm}$.

Re-analyses

For the ten finisher batches in the previous study, smoothing methods 5 and 3 for parenteral and peroral AMs, respectively and farm size adjusted using CHR, were applied to calculate the lifetime AMU for the AMC; aminoglycosides, lincosamides, broad-spectrum penicillins, macrolides, sulfonamides and tetracyclines. The lifetime AMU estimates sum up usage for the entire rearing period per AMC. The lifetime AMU estimates were used as explanatory variables in linear regression re-analyses on the abundance of AMR genes attributed to those AM classes. These results were subsequently compared with the regression results obtained in the previous study (Table 3).

The application of smoothing methods 5 and 3 for parenteral and peroral AMs, respectively, increased the estimated fit of the models (Adj.R^2, AIC and BIC) and therefore potentially explained a larger part of the abundance of AMR genes against aminoglycosides, lincosamides and tetracyclines. For sulfonamides and broad-spectrum penicillins/betalactam, the estimated fit of the models decreased slightly. In contrast, the estimated fit of the model for macrolides decreased substantially (Table 3).

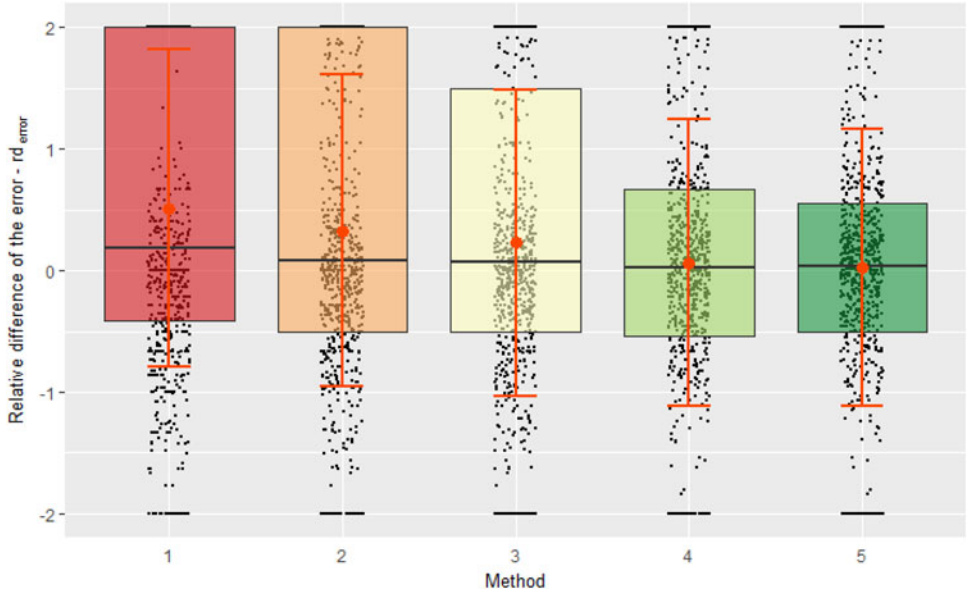


Fig. 3. Boxplots of the relative difference of the error (rd_{error}) for the $Doses_{method}$ 1 to 5 compared with $Doses_{farm}$. The black dots show the individual observations. The orange dots and error bars represent the mean and standard deviation of the rd_{error}.

Downloaded from https://www.cambridge.org/core. IP address: 85.204.133.39, on 04 Mar 2018 at 10:27:15, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268818000134

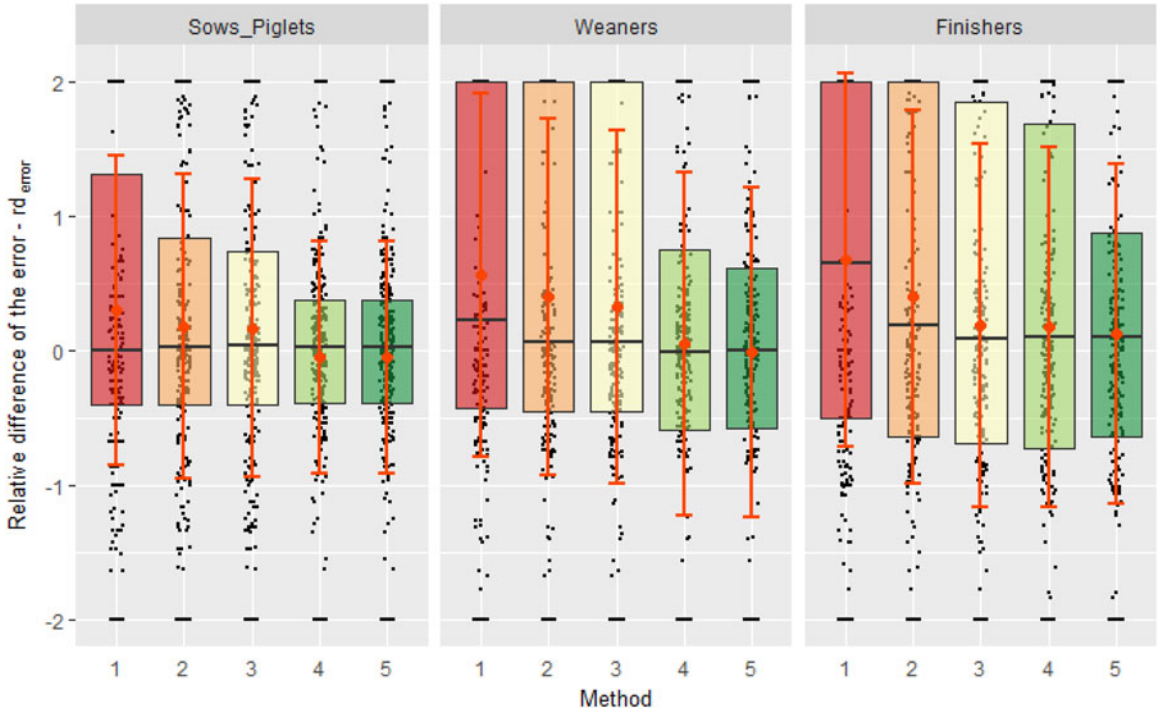


Fig. 4. Boxplots at the age-group level of the relative difference of the error (rd_{error}) for the $Doses_{method}$ 1 to 5 compared with $Doses_{farm}$. The black dots show the individual observations. The orange dots and error bars represent the mean and standard deviation of the rd_{error}.

When the β-coefficient estimate of the model comprising methods 3 and 5 was adjusted in relation to the population ρ_{xx}, the β-coefficient increased by 49%.

The model comprising methods 3 and 5 combined was further evaluated based on alterations of the biomass, model A and B (Table S1 in the supplementary material). The adjustment change of the number of pigs from CHR to PMD had an overall improved effect on tetracyclines, broad-spectrum penicillins, macrolides and lincosamides and the opposite result was found for aminoglycoside and sulfonamides (Table S1 in the supplementary material). The impact of adjusting with PMD rather than CHR related mainly to usage in the age-group; piglets. For the CHR, the

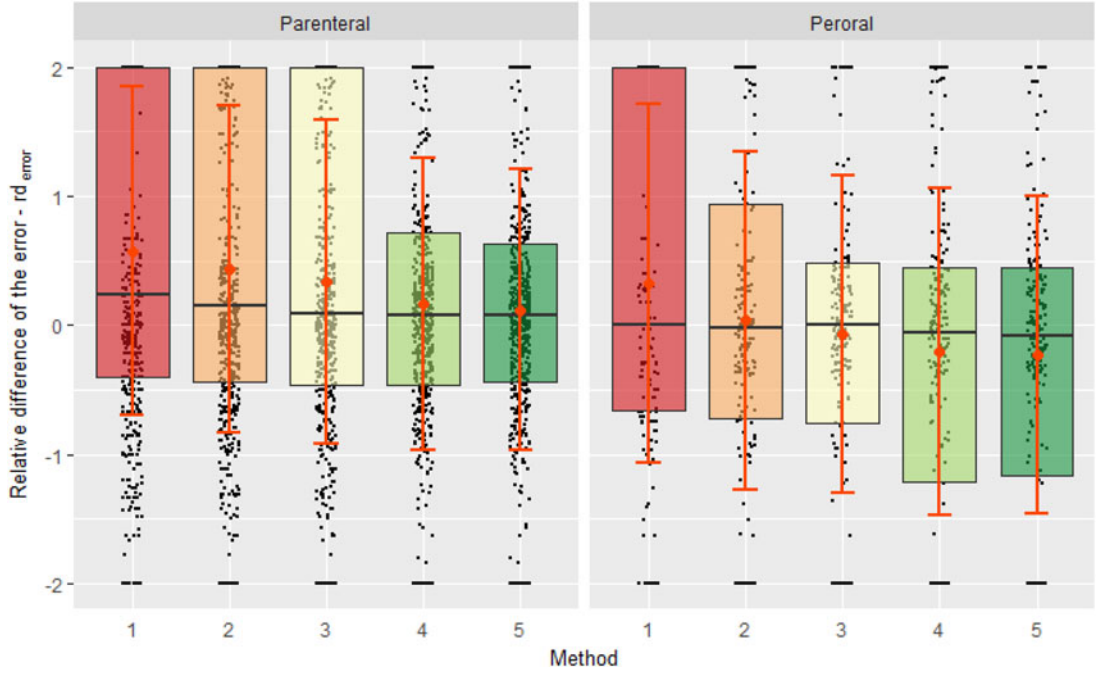


Fig. 5. Boxplots at the dispensing-type level of the relative difference of the error (rd_{error}) for the $Doses_{method}$ 1 to 5 compared with $Doses_{farm}$. The black dots show the individual observations. The orange dots and error bars represent the mean and standard deviation of the rd_{error}.

Downloaded from https://www.cambridge.org/core. IP address: 85.204.133.39, on 04 Mar 2018 at 10:27:15, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268818000134

Table 2. The correlation coefficient (***r***), the Fisher *z* transformed correlation coefficient (r_z) and the reliability coefficient (ρ_{xx}) between the $Doses_{farm}$ and the five $Doses_{method}$ are shown, respectively along with the average-adjustment by farm, age-group, dispensing-type and antimicrobial-class levels, of each coefficient

Method	1	2	3	4	5
r (crude)					
Study population	0.70	0.75	0.76	0.77	0.77
r_z (adjusted by)					
Farm	0.59	0.67	0.70	0.71	0.72
Age-group	0.65	0.70	0.71	0.71	0.71
Dispensing-type	0.70	0.73	0.75	0.76	0.76
Antimicrobial-class	0.73	0.76	0.77	0.81	0.81
ρ_{xx} (crude)					
Study population	0.60	0.65	0.67	0.68	0.68
ρ_{xx} (adjusted by)					
Farm	0.50	0.56	0.60	0.60	0.61
Age-group	0.58	0.62	0.64	0.64	0.64
Dispensing-type	0.60	0.64	0.66	0.67	0.67
Antimicrobial-class	0.57	0.62	0.64	0.68	0.68

number of sows is used as the adjustment factor, resulting in a high number of doses for the piglet age-group, compared with the PMD adjusted estimates (result not shown).

The most notable results were the B models, which had AMU split by dispensing-type into two variables. For these, the estimates of statistical model fit were improved for aminoglycosides, lincosamides and tetracyclines, (Table S1 in the supplementary material).

Discussion

Validation

The completeness of VetStat-records increased from the less detailed method 1 to the more detailed method 5. This was due mainly to the pattern for parenteral usage of AMs, small amounts were used each month and rarely recorded in VetStat. Therefore, the detailed method 5 reflected the true usage of parenteral AMs more closely. Simultaneously, the pattern for peroral usage of AMs caused a reduction in the correctness. According to the farm-records, large amounts of AMs were used for group-treatment within a limited time. As a result, more detailed smoothing caused spurious AMU. Major variations in correctness and completeness could be observed between farms, which could mainly be attributed to dispensing-type and incorrect VetStat-records.

The same pattern for parenteral and peroral AMU affected the relative difference and the correlation coefficient. These became more precise for parenteral usage only when more detailed smoothing methods were applied. Consequently, our results indicate that, due to the differences in usage patterns seen between dispensing-types, the overall most valid method, method 5, for smoothing out the VetStat-records is not applicable for both parenteral and peroral dispensing. For the latter, method 3 is the most valid of the examined methods.

In order for the secondary data, to reflect the true state in a population, high completeness and correctness are required [13, 14]. For the overall most valid method, method 5, the completeness can be categorised as fair [23] and applying different smoothing methods to dispensing-type increased the completeness. In addition, obtaining values of the precision and the impact of the estimate on the statistical association are important for result assessments [15, 21]. A good correlation between farm-records and smoothing method was demonstrated, though it has been pointed out that correlation estimates may not be the optimum method for assessing agreement between methods [15]. In contrast, the standard deviation of the relative difference of the error and the reliability coefficient demonstrated a less precise estimate. Regardless, the reliability coefficient can be used to adjust the β-coefficient in a linear regression, thus the estimate influences the statistical association between AMU and AMR [15, 21].

VetStat gives unique opportunities to study AMU at farm level and its effect on AMR. AM stewardship at farm level and correct recording in VetStat are essential to improve data transformation further. VetStat can provide accurate and precise measurements of AMU through data transformation, which was observed for a number of farms in the validation part. Moreover, VetStat is easily accessible for large parts of a population at farm level [24]. Access to accurate and precise data can then form the basis for establishing knowledgeable guidance and/or adjustments of AMU practices at herd level, with considerably lowering effect on AMU as a result [25]. In addition, the knowledge may also be supportive for detailed risk assessments and trend analyses.

Re-analyses

The results of the re-analyses study indicate that using the alternative smoothing methods produces a better fit regarding the models estimating the effect of AMU on AMR gene abundance. Moreover, when the estimated effects were adjusted by applying the population reliability coefficient, an even higher effect of the lifetime AMU on the abundance of AMR genes was observed, which indicates that the effects estimated in the regression analyses are all underestimated. These results highlight the general importance of valid data in epidemiological studies in order to obtain unbiased quantitative estimates of effects [13–15, 21, 26]. As indicated by the results from the re-analyses, by optimising the utilisation of register data as a proxy for the AMU in pigs and adjusting the regression results obtained based on the results of this validation study, the usage, measured as lifetime AMU, can explain up to 70–80% of the variation in abundance of AMR genes observed between finisher batches.

The deviating result of the effect of macrolide may arise from the time of usage, as the estimated lifetime AMU takes no time-component into account, e.g. usage at different ages has a different impact on the abundance of AMR genes [27–29].

The results of the biomass adjustments according to the CHR and PMD number of pigs revealed that the latter could be a potential substitute for the former. The PMD adjustment was the number of pigs on any given day, estimated from the production of pigs 1 year prior to sampling. This estimate is neutral, as it solely reflects the number of animals being moved, in contrast to the CHR number of pigs, which is a farmer's evaluation of management performance and averages on any given day, thus, more subjective to bias.

Downloaded from https://www.cambridge.org/core. IP address: 85.204.133.39, on 04 Mar 2018 at 10:27:15, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268818000134

Table 3. The results of the linear regression of the previous model and the smoothing methods 3 combined with 5, adjusted by CHR model for usage and abundance of AMR genes to aminoglycosides, lincosamides, broad-spectrum penicillins/betalactam, macrolides, sulfonamides and tetracyclines

	Estimate	SE	*P*-value	Adj.R^2	AIC	BIC
Aminoglycosides						
Model (previous)				0.04	68.64	69.55
(intercept)	18.08 (12.40–23.76)	2.47	0.000			
Aminoglycosides	0.11 (−0.11–0.33)	0.09	0.272			
Model A (methods 3/5)				0.28	65.75	66.66
(intercept)	16.82 (11.73–21.92)	2.21	0.000			
Aminoglycosides	0.19 (−0.02–0.39)	0.09	0.070			
Lincosamides						
Model (previous)				0.20	89.22	90.12
(intercept)	53.51 (36.73–70.29)	7.28	0.000			
Lincosamides	0.68 (−0.19–1.54)	0.38	0.109			
Model A (methods 3/5)				0.51	84.31	85.22
(intercept)	52.54 (40.50–64.59)	5.22	0.000			
Lincosamides	0.64 (0.18–4.28)	0.20	0.012			
Penicillins (broad) – Betalactam resistance						
Model (previous)				0.45	90.26	91.17
(intercept)	52.55 (36.01–69.09)	7.17	0.000			
Penicillins (broad)	0.71 (0.15–1.27)	0.24	0.020			
Model A (methods 3/5)				0.31	92.54	93.45
(intercept)	53.41 (34.40–72.42)	8.24	0.000			
Penicillins (broad)	0.56 (−0.01–1.12)	0.25	0.054			
Macrolides						
Model (previous)				0.66	108.03	108.93
(intercept)	58.34 (−5.52–122.20)	27.69	0.068			
Macrolides	1.82 (0.85–2.78)	0.42	0.002			
Model A (methods 3/5)				0.15	117.38	118.29
(intercept)	105.03 (18.77–191.29)	45.53	0.023			
Macrolides	0.86 (−0.35–1.92)	0.49	0.100			
Sulfonamides						
Model (previous)				0.74	7.14	8.04
(intercept)	−0.16 (−0.50–0.19)	0.15	0.327			
Sulfonamides	0.02 (0.01–0.03)	0.00	0.001			
Model A (method 5)				0.65	9.99	10.90
(intercept)	−0.11 (−0.50–0.29)	0.17	0.552			
Sulfonamides	0.02 (0.01–0.03)	0.00	0.003			
Tetracyclines						
Model (previous)				0.35	108.83	109.74
(intercept)	346.13 (294.36–397.89)	22.45	0.000			
Tetracyclines	0.92 (0.04–1.79)	0.38	0.042			
Model A (methods 3/5)				0.66	102.48	103.38
(intercept)	334.75 (297.17–372.33)	16.30	0.000			
Tetracyclines	0.88 (0.41–1.36)	0.21	0.003			

Downloaded from https://www.cambridge.org/core. IP address: 85.204.133.39, on 04 Mar 2018 at 10:27:15, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268818000134

Conclusions

Based on the validation results, it can be concluded that the VetStat database can be used for refined data transformation to improve accuracy and precision to reflect 'true' AMU at the farm level. Furthermore, the reliability coefficients show that the calculations of the daily amount of AMs used per pig underestimate the usage independent of method.

The knowledge obtained was used to re-calculate lifetime AMU, which in linear regression models provided an overall more beneficial effect on the estimates of statistical model fit than the previous calculation of lifetime AMU. The linear models can be compared only in terms of estimates of statistical model fit, whereas the coefficient estimates should be interpreted with caution due to the limited number of finisher batches in the study.

The PMD could represent an alternative to the CHR for biomass adjustment or should be used to cross-validate the CHR.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0950268818000134.

Acknowledgements. We are very grateful to all the farmers who participated in this study for their help in this important area of research.This work was supported by the Danish Ministry of Food, Agriculture and Fisheries.

Conflicts of interest. None to declare.

References

1. **WHO**. *The Evolving Threat of Antimicrobial Resistance – Options for Action*. World Health Organization, 2012. Available at http://apps.who.int/iris/bitstream/10665/44812/1/9789241503181_eng.pdf (Accessed 1 October 2017).
2. **Levy SB and Marshall B** (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine* **10**(Suppl. 12), 122–129.
3. **Marshall BM and Levy SB** (2011) Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews* **24**, 718–733.
4. **Holmes AH, *et al.*** (2016) Understanding the mechanisms and drivers of antimicrobial resistance. *The Lancet* **387**, 176–187.
5. **Collineau L, *et al.*** (2017) Guidance on the selection of appropriate indicators for quantification of antimicrobial usage in humans and animals. *Zoonoses and Public Health* **64**, 165–184.
6. **European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption**. Sales of veterinary antimicrobial agents in 29 European countries in 2014. Sixth ESVAC report 2016, (EMA/61769/2016). Available at http://www.ema.europa.eu/docs/en_GB/document_library/Report/2016/10/WC500214217.pdf (Accessed 1 October 2017).
7. **Stege H, *et al.*** (2003) VETSTAT – the Danish system for surveillance of the veterinary use of drugs for production animals. *Preventive Veterinary Medicine* **57**, 105–115.
8. **Dupont N and Stege H**. Vetstat-Monitoring usage of antimicrobials in animals. 2013. Available at http://www.icar.org/documents/aarhus_2013/Manuscripts/Dupont.pdf (Accessed 1 October 2017).
9. **Andersen VD, *et al.*** (2017) The association between measurements of antimicrobial use and resistance in the faeces microbiota of finisher batches. *Epidemiology and Infection* **145**, 2827–2837.
10. **Vieira AR, *et al.*** (2009) Association between tetracycline consumption and tetracycline resistance in *Escherichia coli* from healthy Danish slaughter pigs. *Foodborne Pathogens and Disease* **6**, 99–109.
11. **Jensen VF, *et al.*** (2006) Correlation between apramycin and gentamicin use in pigs and an increasing reservoir of gentamicin-resistant *Escherichia coli*. *Journal of Antimicrobial Chemotherapy* **58**, 101–107.
12. **Aarestrup FM** (1999) Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. *International Journal of Antimicrobial Agents* **12**, 279–285.
13. **Emanuelson U and Egenvall A** (2014) The data – sources and validation. *Preventive Veterinary Medicine* **113**, 298–303.
14. **Sørensen HT, Sabroe S and Olsen J** (1996) A framework for evaluation of secondary data sources for epidemiological research. *International Journal of Epidemiology* **25**, 435–442.
15. **Bland JM and Altman DG** (2010) Statistical methods for assessing agreement between two methods of clinical measurement. *The Lancet* **47**, 307–310.
16. **Dupont N, *et al.*** Improving institutional memory on challenges and methods for estimation of pig herd antimicrobial exposure based on data from the Danish Veterinary Medicines Statistics Program (VetStat). 2017. Available at http://arxiv.org/abs/1705.08663 (Accessed 1 October 2017).
17. **Jensen VF, Jacobsen E and Bager F** (2004) Veterinary antimicrobial-usage statistics based on standardized measures of dosage. *Preventive Veterinary Medicine* **64**, 201–215.
18. **SEGES**. ANNUAL REPORT 2014. 2015. Available at http://eng.vsp.lf.dk/~/media/Files/PDF-AarsberetningVSPEnglish/Årsberetning2014_UK.pdf (Accessed 1 October 2017).
19. **Callens B, *et al.*** (2015) Presence of antimicrobial resistance and antimicrobial Use in sows are risk factors for antimicrobial resistance in their offspring. *Microbial Drug Resistance* **21**, 50–58.
20. **Corey DM, Dunlap WP and Burke MJ** (1998) Averaging correlations: expected values and bias in combined Pearson *r*s and Fisher's *z* transformations. *The Journal of General Psychology* **125**, 245–261.
21. **Armstrong BG** (1998) Effect of measurement error on epidemiological studies of environmental and occupational exposures. *Occupational and Environmental Medicine* **55**, 651–656.
22. **Munk P, *et al.*** (2016) A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds. *Journal of Antimicrobial Chemotherapy* **72**, 385–392.
23. **Mörk MJ, *et al.*** (2010) Validation of a national disease recording system for dairy cattle against veterinary practice records. *Preventive Veterinary Medicine* **93**, 183–192.
24. **Birkegård AC, *et al.*** (2017) Computational algorithm for lifetime exposure to antimicrobials in pigs using register data – The LEA algorithm. *Preventive Veterinary Medicine* **146**, 173–180.
25. **Speksnijder DC, *et al.*** (2015) Reduction of veterinary antimicrobial use in the Netherlands. The Dutch success model. *Zoonoses and Public Health* **62**, 79–87.
26. **Dohoo IR** (2014) Bias – is it a problem, and what should we do? *Preventive Veterinary Medicine* **113**, 331–337.
27. **Dunlop RH, *et al.*** (1998) Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada. *Preventive Veterinary Medicine* **34**, 283–305.
28. **Rosengren LB, *et al.*** (2007) Associations between feed and water antimicrobial use in farrow-to-finish swine herds and antimicrobial resistance of fecal *Escherichia coli* from grow-finish pigs. *Microbial Drug Resistance* **13**, 261–270.
29. **Varga C, *et al.*** (2009) Associations between reported on-farm antimicrobial use practices and observed antimicrobial resistance in generic fecal *Escherichia coli* isolated from Alberta finishing swine farms. *Preventive Veterinary Medicine* **88**, 185–192.

Downloaded from https://www.cambridge.org/core. IP address: 85.204.133.39, on 04 Mar 2018 at 10:27:15, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0950268818000134

4.2 Supplementary material

Table 4.2.1. The estimates, standard error (SE) and p-value of the coefficients of the previous method (yellow marking), smoothing methods 3, 5, 3 combined with 5 as one and as two variables, farm size adjusted as number of pigs per day given CHR (model A, green marking)), and PMD (model B, blue marking), of linear regression models of aminoglycosides, lincosamides, broad-spectrum penicillins/beta-lactams, macrolides, sulfonamides, and tetracyclines usage and resistance. In addition, the information measures, adjusted R-squared *(Adj.R^2)*, Akaike information criterion (AIC) and Bayesian information criterion (BIC) for each model.

	Estimates			SE	p-value	*Adj.R^2*	AIC	BIC
Aminoglycosides (Ami.)								
Model (previous)						0.04	68.64	69.55
(intercept)	18.08	(12.40 -	23.76)	2.47	0.000			
Ami.	0.11	(-0.11 -	0.33)	0.09	0.272			
Model A (method 3)						0.21	66.67	67.58
(intercept)	17.19	(11.93 -	22.45)	2.28	0.000			
Ami.	0.17	(-0.04 -	0.38)	0.09	0.101			
Model A (method 5)						0.25	66.13	67.04
(intercept)	17.02	(11.89 -	22.15)	2.23	0.000			
Ami.	0.17	(-0.03 -	0.37)	0.09	0.079			
Model A (methods 3 and 5)						0.28	65.75	66.66
(intercept)	16.82	(11.73 -	21.92)	2.21	0.000			
Ami.	0.19	(-0.02 -	0.39)	0.09	0.070			
Model A (methods 3 and 5)						0.57	61.29	62.51
(intercept)	12.82	(7.29 -	18.34)	2.34	0.003			
Ami. parenteral	0.90	(0.21 -	1.59)	0.29	0.018 *			
Ami. peroral	-0.10	(-0.41 -	0.21)	0.13	0.483			
Model B (method 3)						0.10	67.98	68.88
(intercept)	17.90	(12.51 -	23.28)	2.33	0.000			
Ami.	0.16	(-0.10 -	0.43)	0.12	0.192			
Model B (method 5)						0.12	67.76	68.67
(intercept)	17.77	(12.40 -	23.14)	2.32	0.000			
Ami.	0.17	(-0.09 -	0.43)	0.11	0.172			
Model B (methods 3 and 5)						0.14	67.61	68.72
(intercept)	17.66	(12.28 -	23.04)	2.33	0.000			
Ami.	0.18	(-0.09 -	0.44)	0.11	0.159			
Model B (methods 3 and 5)						0.62	59.99	61.20
(intercept)	11.16	(5.31 -	17.01)	2.47	0.003			
Ami. parenteral	1.83	(0.66 -	3.01)	0.50	0.009 *			
Ami. peroral	-0.16	(-0.46 -	0.14)	0.13	0.239			

	Estimates			SE	p-value	*Adj R²*	AIC	BIC
Lincosamides (Lin.)								
Model (previous)						0.20	89.22	90.12
(intercept)	53.51	(36.73 -	70.29)	7.28	0.000			
Lin.	0.68	(-0.19 -	1.54)	0.38	0.109			
Model A (method 3)						0.49	84.72	85.63
(intercept)	52.11	(39.57 -	64.66)	5.44	0.000			
Lin.	0.62	(0.16 -	1.07)	0.20	0.015 *			
Model A (method 5)						0.51	84.40	85.31
(intercept)	52.59	(40.49 -	64.68)	5.24	0.000			
Lin.	0.64	(0.18 -	1.10)	0.20	0.013 *			
Model A (methods 3 and 5)						0.51	84.31	85.22
(intercept)	52.54	(40.50 -	64.59)	5.22	0.000			
Lin.	0.64	(0.18 -	1.10)	0.20	0.012 *			
Model A (methods 3 and 5)						0.63	82.24	83.45
(intercept)	58.24	(45.29 -	71.18)	5.48	0.000			
Lin. parenteral	-0.20	(-1.33 -	0.94)	0.48	0.689			
Lin. peroral	2.23	(0.18 -	4.28)	0.86	0.037 *			
Model B (method 3)						0.58	82.66	83.57
(intercept)	52.19	(41.18 -	63.21)	4.78	0.000			
Lin.	0.81	(0.30 -	1.31)	0.22	0.006 *			
Model B (method 5)						0.56	83.26	84.17
(intercept)	52.98	(41.85 -	64.12)	4.82	0.000			
Lin.	0.78	(0.27 -	1.30)	0.22	0.008 *			
Model B (methods 3 and 5)						0.57	83.09	84.00
(intercept)	52.85	(41.79 -	63.92)	4.53	0.000			
Lin.	0.79	(0.28 -	1.30)	0.11	0.007 *			
Model B (methods 3 and 5)						0.63	82.24	83.45
(intercept)	57.49	(44.73 -	70.25)	5.40	0.000			
Lin. parenteral	-0.17	(-1.74 -	1.40)	0.66	0.807			
Lin. peroral	2.79	(-0.36 -	5.94)	1.33	0.075 *			

	Estimates			SE	p-value	*Adj R^2*	AIC	BIC
Penicillins (Pen.) (broad) – Beta-lactam resistance								
Model (previous)						0.45	90.26	91.17
(intercept)	52.55	(36.01 -	69.09)	7.17	0.000			
Pen. (broad)	0.71	(0.15 -	1.27)	0.24	0.020 *			
Model A (method 3)						0.36	91.87	92.78
(intercept)	53.74	(35.87 -	71.60)	7.75	0.000			
Pen. (broad)	0.54	(0.03 -	1.04)	0.22	0.040 *			
Model A (method 5)						0.49	89.65	90.56
(intercept)	49.48	(32.15 -	66.82)	7.52	0.000			
Pen. (broad)	0.76	(0.19 -	1.33)	0.25	0.015 *			
Model A (methods 3 and 5)						0.31	92.54	93.45
(intercept)	53.41	(34.40 -	72.42)	8.24	0.000			
Pen. (broad)	0.56	(-0.01 -	1.12)	0.25	0.054			
Model A (methods 3 and 5)						0.35	92.74	93.95
(intercept)	58.31	(36.88 -	79.81)	9.08	0.000			
Pen.(broad) parenteral	-0.31	(-2.14 -	1.52)	0.77	0.700			
Pen.(broad) peroral	0.86	(0.02 -	1.70)	0.35	0.045 *			
Model B (method 3)						0.50	89.38	90.28
(intercept)	53.89	(38.81 -	68.97)	6.54	0.000			
Pen. (broad)	0.70	(0.19 -	1.21)	0.22	0.013 *			
Model B (method 5)						0.62	86.64	87.55
(intercept)	50.52	(36.59 -	64.46)	6.04	0.000			
Pen. (broad)	0.89	(0.37 -	1.42)	0.23	0.004 *			
Model B (methods 3 and 5)						0.49	89.66	90.57
(intercept)	53.41	(37.86 -	68.96)	6.74	0.000			
Pen. (broad)	0.73	(0.18 -	1.27)	0.24	0.015 *			
Model B (methods 3 and 5)						0.54	89.16	90.37
(intercept)	58.12	(41.14 -	75.10)	7.18	0.000			
Pen.(broad) parenteral	-0.53	(-2.72 -	1.65)	0.92	0.581			
Pen.(broad) peroral	0.94	(0.30 -	1.58)	0.27	0.010 *			

	Estimates		SE	p-value	*Adj* R^2	AIC	BIC
	Macrolides (Mac.)						
Model (previous)					0.66	108.03	108.93
(intercept)	58.34	(-5.52 - 122.20)	27.69	0.068			
Mac.	1.82	(0.85 - 2.78)	0.42	0.002 *			
Model A (method 3)					0.23	116.30	117.20
(intercept)	101.54	(14.46 - 188.63)	37.76	0.028			
Mac.	0.94	(-0.18 - 2.06)	0.49	0.090			
Model A (method 5)					0.19	116.89	117.79
(intercept)	110.40	(25.87 - 194.93)	36.67	0.017			
Mac.	0.76	(-0.21 - 1.76)	0.43	0.118			
Model A (methods 3 and 5)					0.15	117.38	118.29
(intercept)	105.03	(18.77 - 191.29)	45.53	0.023			
Mac.	0.86	(-0.35 - 1.92)	0.49	0.100			
Model A (methods 3 and 5)					0.28	116.30	117.51
(intercept)	121.76	(32.19 - 211.57)	37.93	0.015			
Mac. parenteral	-0.08	(-2.06 - 1.89)	0.83	0.923			
Mac. peroral	1.04	(-0.05 - 2.13)	0.46	0.059			
Model B (method 3)					0.33	114.97	115.87
(intercept)	99.51	(22.51 - 176.51)	33.39	0.018			
Mac.	1.24	(0,01 - 2.47)	0.53	0.048 *			
Model B (method 5)					0.31	115.25	116.16
(intercept)	101.96	(24.79 - 179.14)	33.47	0.016			
Mac.	1.17	(-0.03 - 2.38)	0.52	0.055			
Model B (methods 3 and 5)					0.34	114.86	115.77
(intercept)	98.74	(21.90 - 175.60)	33.33	0.018			
Mac.	1.23	(0.03 - 2.44)	0.52	0.046 *			
Model B (methods 3 and 5)					0.27	116.40	117.62
(intercept)	112.76	(12.03 - 213.48)	42.60	0.033			
Mac. parenteral	0.28	(-3.85 - 4.41)	1.75	0.875			
Mac. peroral	1.22	(-0.07 - 2.51)	0.55	0.060			

	Estimates			SE	p-value		*Adj R^2*	AIC	BIC
Sulfonamides (Sul.)									
Model (previous)							0.74	7.14	8.04
(intercept)	-0.16	(-0.50 -	0.19)	0.15	0.327				
Sul.	0.02	(0.01 -	0.03)	0.00	0.001	*			
Model A (method 3)							0.70	8.23	9.14
(intercept)	-0.08	(-0.42 -	0.26)	0.15	0.593				
Sul.	0.02	(0.01 -	0.03)	0.00	0.001	*			
Model A (method 5)							0.65	9.99	10.90
(intercept)	-0.11	(-0.50 -	0.29)	0.17	0.552				
Sul.	0.02	(0.00 -	0.03)	0.00	0.003	*			
Model B (method 3)							0.49	13.84	14.75
(intercept)	0.00	(-0.45 -	0.45)	0.19	0.997				
Sul.	0.04	(0.01 -	0.03)	0.01	0.015	*			
Model B (method 5)							0.50	13.63	14.54
(intercept)	-0.04	(-0.51 -	0.42)	0.20	0.833				
Sul.	0.04	(0.00 -	0.06)	0.01	0.014	*			

	Estimates			SE	p-value		*Adj* R^2	AIC	BIC
	Tetracyclines (Tet.)								
Model (previous)							0.35	108.83	109.74
(intercept)	346.13	(294.36 -	397.89)	22.45	0.000				
Tet.	0.92	(0.04 -	1.79)	0.38	0.042	*			
Model A (method 3)							0.69	101.39	102.30
(intercept)	332.60	(296.63 -	368.56)	15.60	0.000				
Tet.	0.91	(0.45 -	1.36)	0.20	0.001	*			
Model A (method 5)							0.72	100.36	101.27
(intercept)	331.55	(297.38 -	365.73)	14.82	0.000				
Tet.	1.03	(0.55 -	1.52)	0.21	0.001	*			
Model A (methods 3 and 5)							0.66	102.48	103.38
(intercept)	334.75	(297.17 -	372.33)	16.30	0.000				
Tet.	0.88	(0.41 -	1.36)	0.21	0.003	*			
Model A (methods 3 and 5)							0.66	103.05	104.27
(intercept)	329.04	(288.51 -	369.57)	17.14	0.000				
Tet. parenteral	1.41	(0.12 -	2.70)	0.55	0.037	*			
Tet. peroral	0.77	(0.21 -	1.33)	0.24	0.014	*			
Model B (method 3)							0.71	100.64	101.55
(intercept)	339.01	(306.89 -	371.13)	13.93	0.000				
Tet.	0.87	(0.45 -	1.28)	0.18	0.001	*			
Model B (method 5)							0.74	99.38	100.29
(intercept)	337.36	(306.98 -	367.73)	13.17	0.000				
Tet.	1.00	(0.56 -	1.44)	0.19	0.000	*			
Model B (methods 3 and 5)							0.65	102.75	103.65
(intercept)	340.65	(307.23 -	274.06)	14.49	0.000				
Tet.	0.93	(0.42 -	1.28)	0.19	0.002	*			
Model B (methods 3 and 5)							0.82	96.69	97.90
(intercept)	326.85	(298.12 -	355.57)	12.15	0.000				
Tet. parenteral	2.12	(0.94 -	3.31)	0.50	0.004	*			
Tet. peroral	0.71	(0.35 -	1.07)	0.15	0.002	*			

4.3 Discussion

Several studies have assessed CHR, PMD and VetStat in terms of data quality and usability for surveillance (Houe, Gardner and Nielsen, 2011; Dupont *et al.*, 2016, 2017). The objective of this study was not to assess the databases themselves. The objective was to assess how well transformation of VetStat data could mimic the AMU in pigs through rearing, together with an assessment of the impact on the AMU and AMR association of using two different biomass adjustments obtained from the CHR and the PMD databases.

The underlying principles of the lifetime AMU were several unproven assumptions. However, it was key that when a VetStat-record was followed by a new VetStat-record, the amount prescribed was assumed to have been used during the period between records (smoothing). Therefore, taking the periods between VetStat-records into account, five different approaches to transforming data from VetStat were compared with farm-records. The limitation of the validation was the incomplete availability of farm-records equivalent to finisher batches' entire rearing pathways. Instead, records were obtained from farm units with no coherent production enrolled in another study. Consequently, the validation related just to the estimated daily AMU per pig (ADDkg/pig.day), which is the foundation for lifetime AMU.

High accuracy (completeness and correctness) and precision (correlation, relative difference of the error and the reliability coefficient) are required in order for register data, to reflect the true state in a population (Sørensen, Sabroe and Olsen, 1996; Armstrong, 1998; Bland and Altman, 2010; Emanuelson and Egenvall, 2014). The accuracy and precision estimates describes the measurement error of the daily AMU estimate. In addition, the reliability coefficient can be used to adjust the point estimate of linear regression models using these data, thus providing an estimate of the influence of the measurement error on the statistical association between AMU and AMR.

The validation estimates for accuracy and precision all demonstrated that the intervals in days between VetStat-records should be calculated taking two additional levels (dispensing-type and AM-class) into account, when data were smoothed over a period, and that different smoothing approaches should be employed for parenteral and peroral dispensing, respectively, to obtain the best measure of the daily AMU per pig. The difference observed between the parenteral and peroral smoothing method could be explained by the difference in the prescription pattern and the subsequent farm usage. For the parenteral AMs, they were infrequently prescribed in large amounts and AM-class alterations within a diagnosis seldom occurred. However, in the farms, small amounts were used on a regular monthly basis. For the peroral AMs, they were infrequent prescribed in large amounts, but used irregularly, furthermore, in several cases the amount prescribed (AM product) in connection with a mandatory vet visit had not been used at the time of the next mandatory vet visit, and yet at this visit, the same AM product was prescribed once again. In addition, for the peroral AMs, AM-class alterations within a diagnosis occurred regularly. Any injudicious prescriptions of AMs, e.g. prescriptions without usage of the previous prescribed, has a harmful effect on the precision of the daily usage estimate. In contrast to this, in some study farms the farm-records and VetStat-records

were in almost perfect alignment in terms of accuracy and precision, which shows that when farmers and vets practice prudent AM stewardship (Appendix B - Table B1) the measure of daily AMU improves. In general, independent of smoothing method, the calculated daily AMU underestimated the usage compared with the "true" state, obtained from farm record.

Another issue encountered related to VetStat involved records for one age-group, whereas farm-records revealed usage in two age-groups or usage in a different age-group. It is mandatory to register the correct age-group in VetStat (Executive order 1353/2017), but the DVFA also accepts that prescriptions assigned to one age-group can be used for several age-group in a farm (DVFA, 2017). This counterproductive practice will reduce both the accuracy and precision of the daily AMU, by wrongly assigning usage in one age-group at the expenses of reduced usage in another. Since it is legal, the practice could be common, however, besides the encountered praxis in this study, the authors have no knowledge of the extent of the praxis in Danish pig production (Appendix B – Table B2). Furthermore, the table provides a little insight into the differences between AMU in piglets and sows, e.g. aminoglycosides, aminoglycosides combined with narrow-spectrum penicillins, colistin, lincosamides combined with spectinomycin and long-lasting macrolides were only used for piglets, whereas narrow-spectrum penicillins and tetracyclines were only used for sows, knowledge which may assist in attempts to separating AMU between the two.

With the purpose of gathering knowledge of the differences between the vets' instructions on AMU and those recorded in VetStat, the instructed dose was compared with the standard ADD and the duration was compared with the recommended duration in the product catalogue of the Danish Veterinary Medical Industry. A vet instruction guides the farmer on AMU, i.e. disease symptom to be detected before initiating treatment, treatment dose per kg pig per day and duration of the treatment in days. The dose and duration evaluation revealed that the vets over-dosed the parenteral AMs, in particular, the long-lasting parenteral AMs, while the peroral AMs were slightly under-dosed (Appendix B - Fig. B3 – B4). A finding that other studies have also demonstrated (Timmerman *et al.*, 2006; Callens *et al.*, 2012; Trauffler *et al.*, 2014). An overview of the diseases that the AMs were prescribed for showed similarities between farms, e.g. long-acting extended penicillin (amoxicillin) was the only product used to treat omphalitis, single drug peroral aminoglycosides and colistin were only used to treat diarrhea, while parenteral aminoglycosides in combination with narrow-spectrum penicillins were only used to treat arthritis (Appendix B – Fig. B5). Part of the treatment instructions for specific diagnosis did not change over time, which could indicate that some treatments are standard management procedures. Callens *et al*. (2012) found that 93% of group-treatments were preventive and often lacked a precise diagnosis. They argued that highly specialised farms often have standardised management procedures to prevent production losses, and that these standard treatments are easier and less labour intensive to implement than treating clinically diseased animals with concomitant losses in production. The higher usage was attributed to treatments during critical management procedures, such as castration, tail docking and weaning, because the farmer foresees pigs becoming diseased during these circumstances. This practice is not unfamiliar in Danish pig farms, which face the same consideration of optimising production by reducing foreseen diseases (Jørgensen *et al.*, 2007; Nielsen, 2015).

When the alternative smoothing methods were employed in the re-analyses of 10 finisher batches from a previous study, the results produced an overall better statistical estimate of model fit of the regression analyses estimating the effect of AMU on AMR gene abundance compared with the previous models. Moreover, when the reliability coefficients were employed to the point estimate of the regression analyses, they increased by 49%. Subsequently demonstrating that the measurement error were biasing the effect estimates towards zero. The deviating result of the effect of macrolide may arise from the time of usage. This predicament might be solved by including the weight of the pigs, which in the first study beneficially altered the effect of macrolide usage on the occurrence of macrolide resistance, thus the variation in macrolide resistance that macrolide usage was able to explain changed from 66% to 72% (Appendix A - Fig. A1).

Also, in this study, two different biomass adjustments based on data from the CHR and the PMD databases were assessed. The former database is commonly used in Danish research (Emborg *et al.*, 2007; Vieira *et al.*, 2009; Vigre *et al.*, 2010). However, in recent years an increased deviation between the number of pigs in farms and the number of pigs delivered for slaughter has been noticed, which was also demonstrated in this study by comparing the number of animals obtained from the two databases (Appendix B - Fig. B6). The comparison between the numbers of pigs revealed that when the number of piglets was calculated based on the production data from the PMD, per se exceeded the number of sows registered in the CHR, which may provide a better biomass adjustment in the calculation of lifetime AMU. The number of weaners was higher and the number of finishers was lower in the CHR than the number of weaners and finishers in the PMD. The PMD biomass adjustment may be less biased, as it solely reflects the number of animals being moved, in contrast with the CHR number of pigs, which is a farmer's evaluation of management performance and averages on any given day, and is therefore more subject to bias.

Then, based on how much variation of AMR the AMU could explain, comparison between the number of pigs at any given day obtained from the CHR and the PMD revealed that using the PMD in regression analyses gave improved statistical models fit estimates. The impact of adjusting with the PMD rather than the CHR related mainly to usage in piglets. When the number of sows (CHR) was used as the biomass adjustment, the estimated number of daily AMU in piglets rose, compared with the PMD adjusted estimates. A high number of daily doses in the piglet rearing period can distort the AMU-AMR relationship by putting too much emphasis on this period. Generally, the results of the biomass adjustments revealed that the PMD performed better than the CHR, suggesting that the PMD provides a more accurate and reliable estimate of the number of pigs at a farm.

The improved results of the regression analyses led to the analyses of the effect of parenteral and peroral AMU on the AMR abundance of the ten finisher batches. Besides the difference between dispensing-types per se, the two variables also comprise a somewhat semi-representation of time of usage, because parenteral AMs primarily were used for sows and piglets, whereas peroral AMs were primarily used for weaners and finishers (Jensen *et al.*, 2014; Dupont *et al.*, 2016). The results of these analyses revealed that the effect estimates were indeed affected differently by dispensing-type,

and to some degree the strongest effects were in alignment with the time of usage observed for the batches, i.e. parenteral aminoglycosides were used primarily for piglets – sows and tetracyclines were used in all age-groups independent of dispensing-type (Table 3.2.1. and Table 4.2.1).

5. Objective III

5.1 Manuscript III

Predicting effects of interventions targeting antimicrobial usage on antimicrobial resistance abundance in the gut microbiome of finishers

V. D. Andersen[a1], F. M. Aarestrup[a1], P. Munk[a], M. S. Jensen[a], L. V. de Knegt[a], V. Bortolaia[a], B. E. Knudsen[a], O. Lukjancenko[a], A. C. Birkegård[b], and H. Vigre[a1]

[a] The National Food Institute, Technical University of Denmark, Lyngby, Denmark
[b] The National Veterinary Institute, Technical University of Denmark, Lyngby, Denmark[1]

[1] Corresponding authors.

Submitted February 2018 to Proceedings of the National Academy of Sciences (PNAS)

Predicting effects of interventions targeting antimicrobial usage on antimicrobial resistance abundance in finisher's gut microbiomes

V. D. Andersen[a1], F. M. Aarestrup[a1], P. Munk[a], M. S. Jensen[a], L. V. de Knegt[a], V. Bortolaia[a], B. E. Knudsen[a], O. Lukjancenko[a], A. C. Birkegård[b], H. Vigre[a1]

[a] *The National Food Institute, Technical University of Denmark, Lyngby, Denmark*
[b] *The National Veterinary Institute, Technical University of Denmark, Lyngby, Denmark*

[1]Corresponding author:
Vibe Dalhoff Andersen
Research Group for Genomic Epidemiology
Technical University of Denmark
Søltoft Plads, DK-2800 Kgs. Lyngby, Denmark
Tel: +45 35 88 71 13, E-mail: vdan@food.dtu.dk
[1]To whom correspondence may be addressed. Email: vdan@food.dtu.dk, fmaa@food.dtu.dk or hvig@food.dtu.dk.

Abstract
It is generally accepted that production animals contributes to the burden of antimicrobial resistance (AMR) in humans. To curb the increased occurrence of AMR in food animals requires in-depth knowledge of the quantitative relationship between antimicrobial usage (AMU) and AMR to achieve desired resistance reductions from interventions targeted AMU. The relationships between lifetime AMU in finisher batches close to slaughter and the abundance of AMR genes (AMR abundance) in their gut microbiome were quantified using linear regression. These results and the national AMU in pigs were included in a predictive model that allowed testing of different lifetime AMU scenarios for finishers slaughtered in Denmark. Three different scenarios of lifetime AMU were simulated in the model. When all tetracycline usage were ceased, the aminoglycoside, lincosamide and tetracycline resistance were reduced by 4-42%, 0-8% and 9-18%, respectively. When the peroral tetracycline usage of the 10% highest users were replaced with peroral macrolide usage, the tetracycline resistance was reduced by 1-2% and the macrolide and MLSb resistance increased by 5-8%. When all extended-spectrum penicillin usage were replaced with parenteral lincosamide usage, the beta-lactam resistance reduced by 2-7%, but the lincosamide usage and resistance increased by 200% and 13-42%, respectively. Nonetheless, interventions targeting AMU will reduce the overall AMR abundance, though differently depending on the targeted AM-class and provided the reduction in usage of one AM-class is not replaced with usage of another. This study provides a framework for further development, which may assist in reducing AMR thus safeguarding AMs for the future.

Significance
Being able to predict the quantitative effects of interventions targeting AMU on AMR, comprises a valuable guidance tool for authorities and stakeholders. Quantified relationships between finisher batches lifetime AMU and AMR abundance in their gut microbiome combined with national AMU at farm level were included in a predictive model, which allowed for testing of different AMU scenarios of finishers slaughtered in Denmark. The model shows that large reductions of commonly used AMs led to minor reductions in AMR, and might increase the overall abundance, if reduction of one AM-class is replaced with usage of another AM-class. However, targeted interventions will potentially provide an overall beneficial effect. This study provides a framework for further developments in animal and human settings globally.

\body
Introduction
Antimicrobial resistance (AMR) is considered one of the most harmful threats to global health, and it is widely accepted that the antimicrobial usage (AMU) is the main cause (1, 2). In an attempt to reduce the occurrence of AMR, several AMU stewardship programs have been implemented, but these programs have proven difficulties in introducing major declining effects on the occurrence of AMR, and none of them were able to predict any changes (3, 4).
Even though it is generally accepted that production animals contribute to the burden of AMR among humans (5, 6), less is known about the causes of the emergence and spread of AMR in the food chain and the risk posed to humans (6). In particular, there is a large debate on the contribution of foodborne AMR to human infections (5–7). Consequently, attention towards AMU in production animals has grown immensely during the past decade (5). In several countries, this has led to the establishing of surveillance systems that monitor trends and changes in AMU in animals (8). These monitoring systems has in turn been efficiently applied to facilitate interventions and guidelines for improved antimicrobial (AM) stewardship (3, 4, 9).
Epidemiological studies have established that AMU and AMR in production animals are closely related (6), and it has also been proven that halted usage of a compound, e.g. vancomycin and third-generation cephalosporin will lead to declined occurrence of AMR (10, 11). However, the quantitative relationship between AMU and AMR is not as illuminated, because neither the quantification of AMU nor the characteristics of the AMs, e.g. route of administration, dose, duration of treatment, and concurrent interrelation between AM-compounds have been fully determined in terms of importance for the selection of AMR (12). In addition, most studies conducted so far have focused on only one or a few indicator bacteria, whereas the bulk of relevant AMR genes might be present in the entire gut microbiome. The recent developments in next generation sequencing allow complete quantification of the abundance of AMR genes (AMR abundance) in the entire gut microbiome (13).
Denmark is one of the largest exporters of pork products globally (14), and the total AMU within the Danish pig production is considerable; in 2016, it represented 75% of the total amount of kilogram active substance for animals and it was 57% higher compared to human usage (15). In a public health perspective, the AMU in the pig production in Denmark is worrying therefore, even though the Danish authorities since have launched several initiatives to reduce the usage in pigs.
To provide the means of estimating an association between specific AMU and AMR at national level, two previously conducted studies developed and validated a method based on register-data, which quantified the associations between lifetime AMU of six AM-classes used in the pig production and AMR abundance of these classes for ten finisher batches close to slaughter. Subsequently, a method that measured AMU in finisher batches throughout their lifetime by combining usage in the piglet (sow), weaner and finisher rearing period, independent of rearing site. In the study, the AMR abundance was measured using shotgun metagenomic sequencing, which gave a proportional content of AMR abundance independent of bacteria species (13, 16, 17).
Studies have been published focusing on predicting the effect of AMU changes on the occurrence of AMR (18–21). Most of these studies were based on theoretical data and to the best of the authors' knowledge, none concerning AMR abundance in the gut microbiome of animals. Having precise knowledge of the effect of lifetime AMU of individual AM-classes on all AMR abundances, combined with knowledge of either AMU or AMR for larger parts of a population, allows for the prediction of the overall effect of an intervention targeting AMU in general or for specific AM-classes only in that population, for instance a country (22, 23). Additionally, a modelling framework of the epidemiology of AMR abundance used to describe differences in the gut microbiome of finishers under the influence of AM pressure can also be used to support knowledgeable guidance of AMU practices at farm level.
In this study, first a characterisation of the gut resistome of 83 finisher batches representing the majority of pigs slaughtered in Denmark in 2013 was performed, and through data transformation of register data, the lifetime AMU for these batches were calculated. Based on the data, the quantitative effect of lifetime AMU on the AMR abundance in the gut microbiome of finisher batches close to slaughter were estimated. Then, the AMU for all pig farms at unit level in Denmark was calculated. From the effect estimates and the national data on AMU, a

predictive model was developed, wherein the effect of different scenarios of nationwide reduction in AMU on the AMR abundance in the gut microbiome of finishers close to slaughter in Denmark can be assessed.

Results

Study population

The finisher batches varied in rearing pathways, however, the pathway of the majority of batches were simple, i.e. pigs in a unit originated from the same farm or from one farm only (Fig. S1). Two finisher batches did stand out, due to the complexity of their rearing pathway that included five and seven farms, respectively (Fig. S1).

The parenteral and peroral lifetime AMU of nine AM-classes; aminoglycosides including spectinomycin, extended-spectrum penicillins, lincosamides, macrolides, narrow-spectrum penicillins, pleuromutilins, polymyxins, sulfonamides including trimethoprim and tetracyclines of the 83 finisher batches is presented in Fig. S2. The extended-spectrum penicillins, macrolides, narrow-spectrum penicillins and tetracyclines were the most commonly used AM-classes of parenteral dispensing, whereas the most commonly used AM-classes of peroral dispensing were macrolides, pleuromutilins and tetracyclines. The distribution of parenteral and peroral lifetime AMU per rearing unit revealed that parenteral dispensing was mainly used for piglets, including sows, while the main usage for weaners and finishers were peroral dispensing (Fig. S2). In the scatterplots (Fig. 1), the AMR abundance per AM-class of the 83 finisher batches is shown. Overall, tetracycline resistance was most abundant, followed by macrolide resistance. In contrast, resistance of sulfonamide was scarcely found.

Regression analyses

The model assumptions of constant variance and normality of residuals were evaluated by visually inspection of the diagnostic plots of the models. From this, it was concluded that the assumptions to perform linear regression explaining AMR by the AMU were fulfilled without any kind of data transformation.

Though, for lincosamides, one observation with a Cook's distance higher than one was identified, and when the impact of excluding this observation on the model was assessed, the lifetime usage of lincosamides were still significant, but the β-coefficient of lincosamides increased app. 49% and the R^2 decreased app. 43%. Nonetheless, it was decided to keep the observation in the subsequent analyses. Overall, the results from the bi-square robust regression models indicated that the data did not have any notable influential observations (Fig. 1 and Table S3).

Uni-variable model - Model 1

For all AM-classes, except narrow-spectrum penicillins and sulfonamides, the lifetime usage demonstrated a significant effect on the resistance abundance (Fig. 1). For the significant results, the proportion of observed variation (R^2) in AMR abundance that could be explained by the lifetime usage ranged from 6% (extended-spectrum penicillin on beta-lactam) to 49% (macrolides on MLSb resistance) (Fig. 1). When the estimated models of bi-square robust regressions were added to the scatterplots, the obtained estimates of the fitted models showed to be similar to the estimates obtained in the linear regression models, which indicated that the data did not have any notable influential observations (Fig. 1).

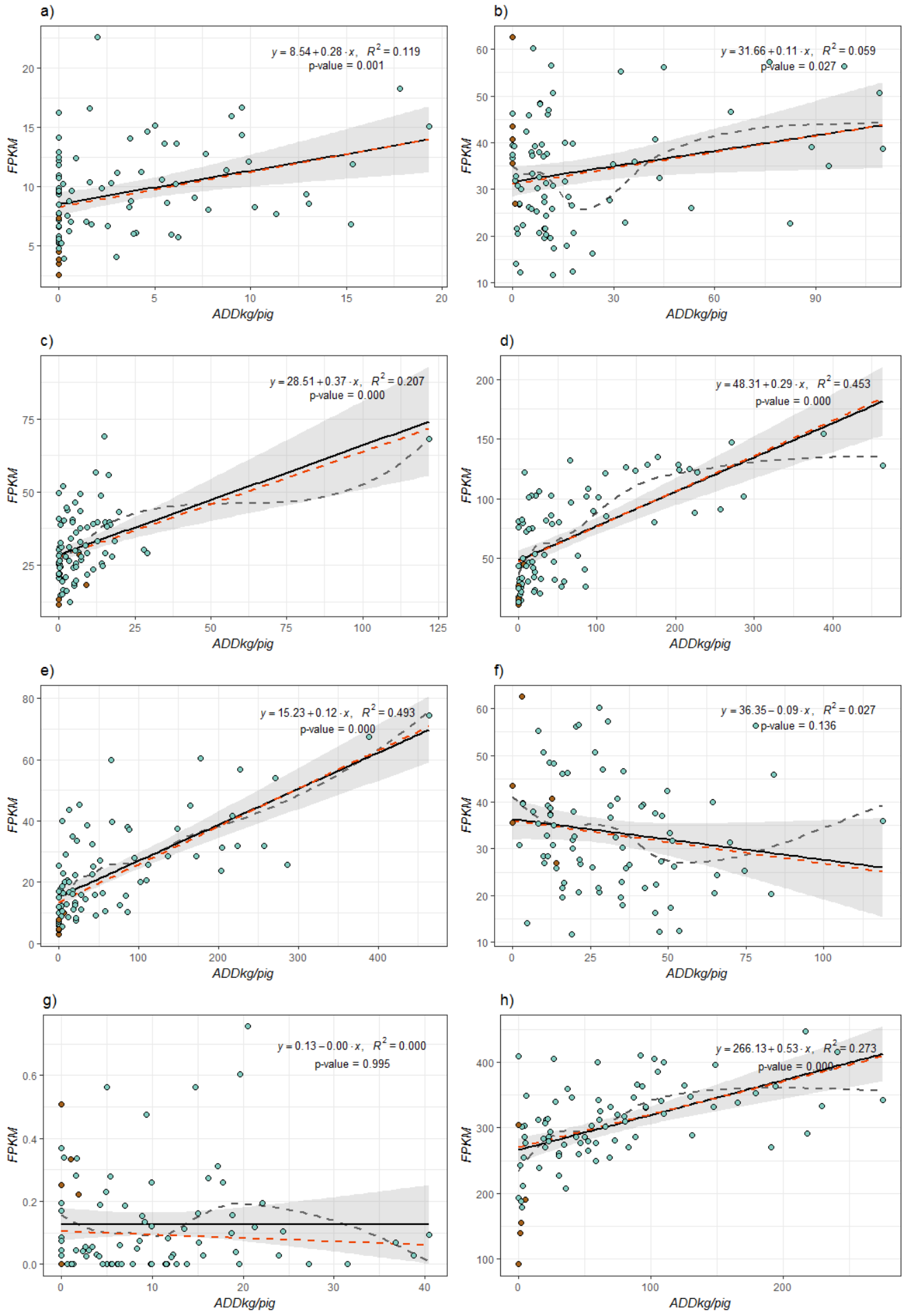

a)
y = 8.54 + 0.28 · x, R² = 0.119
p-value = 0.001
b)
y = 31.66 + 0.11 · x, R² = 0.059
p-value = 0.027
c)
y = 28.51 + 0.37 · x, R² = 0.207
p-value = 0.000
d)
y = 48.31 + 0.29 · x, R² = 0.453
p-value = 0.000
e)
y = 15.23 + 0.12 · x, R² = 0.493
p-value = 0.000
f)
y = 36.35 − 0.09 · x, R² = 0.027
p-value = 0.136
g)
y = 0.13 − 0.00 · x, R² = 0.000
p-value = 0.995
h)
y = 266.13 + 0.53 · x, R² = 0.273
p-value = 0.000
FPKM
ADDkg/pig

Fig. 1. Plotted observations of lifetime AMU (*ADDkg/pig)* against resistance abundance (*FPKM*) for conventional (blue) and organic farms (brown) of; a) aminoglycoside usage and resistance, b) extended-spectrum penicillin usage and beta-lactam resistance, c) lincosamide usage and resistance, d) macrolide usage and resistance, e) macrolide usage and MLSb resistance, f) narrow-spectrum penicillin usage and beta-lactam resistance, g) sulfonamide usage and resistance, and h) tetracycline usage and resistance. In addition, the three regressions of *FPKM* as a function of *ADDkg/pig*; i) LOESS local (dotted grey line), ii) linear with 95% confidence interval (CI) (black line and grey area), and bi-square robust (orange dotted line) together with the function, p-value and R^2 value are shown in each plot.

Model 1 included updated design variables – Model 2

When the design variable, production-type was included in the initial uni-variable models (Model 1), the estimated β-coefficients of the lifetime AMU differed from the β-coefficients of Model 1 by 0% to 19%, thus, it could not be concluded that production-type was an important confounder for the effect of lifetime AMU (Table 1). In the organic farms, the abundance of beta-lactam and sulfonamide resistance was significantly higher, while the abundance of aminoglycoside, lincosamide, macrolide, and tetracycline resistance was significant lower. When the updated design variables; the annual number of suppliers of pigs and the annual number of slaughtered finishers were included in the initial uni-variable models (Model 1), the estimated β-coefficients of the lifetime AMU differed from the β-coefficients of the Model 1 by 0% to 16%. Therefore, it could not be concluded that these variables were important confounders for the effect of lifetime AMU (Table 1).

Multi-variable model at dispensing-type level – Model 3

The multi-variable regression models of the effect of parenteral and peroral lifetime usage of aminoglycosides, extended-spectrum penicillins, lincosamides, macrolides, sulfonamides and tetracyclines on aminoglycoside, beta-lactam, lincosamide, macrolide/MLSb, sulfonamides and tetracycline resistance abundance, respectively, displayed a substantial difference between the parenteral and peroral β-coefficients for all AM-class resistances. Therefore also a substantial difference compared to the β-coefficients of the uni-variable models (Table 1). The β-coefficients of peroral aminoglycosides, parenteral extended-spectrum penicillins and parenteral macrolides were without effect, therefore, significant results were only obtained for parenteral usage of aminoglycosides, for peroral usage of extended-spectrum penicillins and macrolides, and for both parenteral and peroral usage for lincosamides and tetracycline (Table 1). Neither parenteral nor peroral usage of sulfonamides provided any significant results (Tables 1).

Table 1. β-coefficients of the lifetime AMU estimated as total usage (Models 1 - 2) and as parenteral and peroral usage (Models 3 – 4) of aminoglycosides, extended-spectrum penicillins, lincosamides, macrolides, sulfonamides and tetracyclines. Grey numbers of β-coefficients of Models 2 indicate the updated variable(s) were not significant in the regression analyses.

	Model β-coefficient						
	1	2				3	4
		Prod.*	Slaught.†	Suppl.‡	Slaught.-Suppl.		
Aminoglycosides							
Total	0.28	0.24	0.24	0.23	0.19	-	-
Parenteral	-	-	-	-	-	0.34	0.34
Peroral	-	-	-	-	-	0.04§	-
Extended-spectrum penicillins							
Total	0.11	0.13	0.13	0.13	0.13	-	-
Parenteral	-	-	-	-	-	0.00§	-
Peroral	-	-	-	-	-	0.14	0.15
Lincosamides							
Total	0.37	0.36	0.35	0.36	0.36	-	-
Parenteral	-	-	-	-	-	0.65	0.59
Peroral	-	-	-	-	-	0.32	0.32
Macrolides							
Total	0.29	0.28	0.27	0.28	0.37	-	-
Parenteral	-	-	-	-	-	-0.01§	-
Peroral	-	-	-	-	-	0.28	0.28
Macrolides (MLSb)							
Total	0.12	0.11	0.11	0.12	0.12	-	-
Parenteral	-	-	-	-	-	-0.04§	-
Peroral	-	-	-	-	-	0.12	0.11
Sulfonamides							
Total	-0.00§	0.00	0.00	0.00	0.00	-	-
Parenteral	-	-	-	-	-	0.00§	-
Peroral	-	-	-	-	-	-0.00§	-
Tetracyclines							
Total	0.53	0.43	0.46	0.44	0.45	-	-
Parenteral	-	-	-	-	-	0.64	0.68
Peroral	-	-	-	-	-	0.41	0.45

* Production-type
† Annual number of slaughtered finishers
‡ Annual number of suppliers of pigs
§ Variable was not significant in the regression analyses

Multi-variable model included all AM-classes at dispensing-type level and updated design variables - Model 4

When the β-coefficients of the reduced multi-variable regression models were compared to the Model 3 significant β-coefficient of the same AM-class resistance, the β-coefficients of peroral extended-spectrum penicillins, parenteral lincosamides, peroral macrolides (MLSb), parenteral and peroral tetracyclines changed by 7%, 9%, 8%, 6% and 10%, respectively. In contrast, parenteral aminoglycosides, peroral lincosamides and peroral macrolides, did not change (Tables 1). The updates design variables; annual

number of slaughtered finishers and annual number of suppliers of pigs were excluded during model reduction of all AM-class resistances (Table S3).
The significant results of the resistance abundance of the seven AM-classes demonstrated two noticeable outcomes, the overall difference between parenteral and peroral dispensing and the effect of peroral macrolides and both parenteral and peroral tetracyclines on several AMR-classes. In addition, even though the β-coefficient values were minute, the sulfonamide resistance abundance seemed to be affected by peroral polymyxins (Fig. 2 and Table S3).

The obtained estimates of the fitted bi-square robuast regression models changed the significant β-coefficient of parenteral aminoglycosides, parenteral lincosamides and peroral macrolides (MLSb) by 7%, 7% and 6%, respectively, within their respective AM-classes, whereas the β-coefficient of peroral extended-spectrum penicillins, peroral lincosamides, peroral macrolides, parenteral and peroral tetracyclines within their respective AM-classes changed 2% or less (Table S3). Results, which indicate that the estimated effect are robust against outliers.

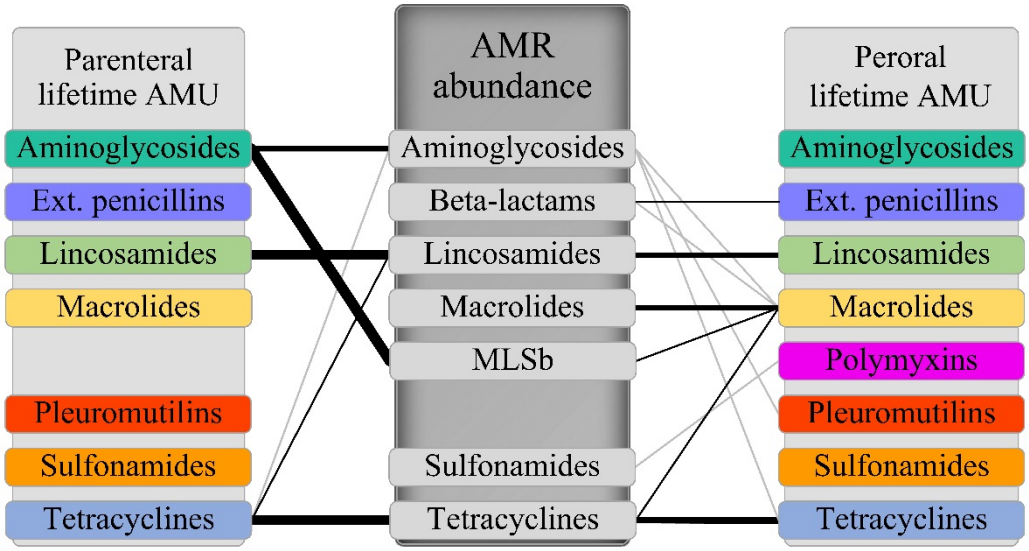


Fig. 2. Results of Model 5 of the effect of parenteral and peroral lifetime AMU of aminoglycosides, extended-spectrum (Ext) penicillins, lincosamides macrolides, pleuromutilins, polymyxins, sulfonamides and tetracyclines on the aminoglycoside, beta-lactam, lincosamide, macrolide, MLSb, sulfonamide and tetracycline resistance abundance. Black lines indicate the main significant result, and thickness is proportional to the relative size of the β-coefficient. Grey lines indicate significant result with β-coefficient less than 0.05.

Uni-variable model at resistance gene level – Model 5.
The multi-variable regression models of the effect of parenteral and peroral lifetime usage of aminoglycosides, extended-spectrum penicillins, lincosamides, macrolides, narrow-spectrum penicillins, pleuromutilins, polymyxins, spectinomycin, sulfonamides, tetracyclines and trimethoprim on each resistance gene of the AM-classes; aminoglycosides, beta-lactams, lincosamides, macrolide/MLSb, sulfonamides and tetracycline, demonstrated that the specific AM-class resistance genes was foremost affected by the same AM-class usage (Fig. S4). Conversely, the abundance of aminoglycoside resistance genes was affected by several AM-classes, which is consistent with the results of Model 4 of aminoglycoside resistance, where it was demonstrated that several AM-classes had an impact on the occurrence (Table S3). In addition, the abundance of MLSb resistance genes, *erm*(B), *erm*(F) and *erm*(G), seemed to be affected by the parenteral aminoglycoside usage, which was in alignment with the MLSb result of Model 4 (Fig. 2 and Table S3). The sulfonamide *sul3* resistance gene was the only AM-class specific gene affected by sulfonamide usage, a finding that differed from the Model 4, where sulfonamide usage had no effect on sulfonamide resistance. Although most of the tetracycline resistance genes were affected by peroral tetracycline usage, several tetracycline resistance genes were affected by other AM-classes. In contrast, *tet*(X) was not affected by tetracycline usage, instead it was affected by the usage of parenteral aminoglycosides, parenteral lincosamides, peroral macrolides and peroral polymyxins (Fig. S4).

Correlation matrix at resistance gene level – Matrix 6
The correlation matrix of the resistance genes of the aminoglycoside, beta-lactam, lincosamide, macrolide, MLSb, sulfonamides and tetracycline classes is presented in Fig. S5. Within the AM-classes; aminoglycosides, beta-lactams and lincosamides, none of the resistance genes were highly correlated. In the macrolide-class, the *mef*(A) gene correlated with the *msr*(D). Within the MLSb-class, the *erm*(B), *erm*(F), *erm*(G), *erm*(Q) and *erm*(T) genes correlated with each other, thus, formed a cluster. For the sulfonamide-class, the *sul*1 gene correlated with the *sul*2 gene. In the tetracycline-class, the *tetA*(P) and *tetB*(P) genes correlated, for that reason, both of them correlated with the *tet*(44) gene. The *tet*(32) gene correlated foremost with the *tet*(40), *tet*(L) and *tet*(W) genes, and the *tet*(40) gene correlated with the *tet*(Q) and *tet*(W) genes (Fig. S5).
Several correlations between genes of different AM-classes were notable. The aminoglycoside *aadE* gene correlated with the lincosamide *lnu*(B) gene, the aminoglycoside *strA* and *strB* genes correlated with the sulfonamide *sul1* and *sul2* genes, the aminoglycoside *ant*(6)-I gene correlated with the tetracycline *tet*(44), *tetA*(P) and *tetB*(P) genes, and the aminoglycoside *aph*(3')-III gene correlated with the tetracycline *tet*(32), *tet*(40), and *tet*(Q) genes. The beta-lactam resistance genes did not correlate with resistance genes of other AM-classes, while the

macrolide *mef*(A) *and msr*(D) gene correlated with most of the MLSb genes and tetracycline-*tet*(X) gene (Fig. S5)

Predictive model

First, the AMU in the farms contributing with finisher batches to the observational study was evenly distributed among the farms contributing with batches used for prediction. For every AM-class at dispensing-type level, within every decile of the 3080 finisher batches used for the prediction model, it was found that 5-15 % of the batches originated from farms, which also contributed with batches in the observational study.

The predicted effect of different lifetime AMU scenarios in the Danish pig production on the overall resistance abundance of different AM-classes is presented in Table 2.

Table 2. Result from the simulation studies predicting the effect in percentages of three different scenarios of lifetime AMU in Danish finisher batches on AMR abundance of different AM-classes in the bathes microbiome close to slaughter at national level.

Scenario	Change in AMR abundance (%)						Change in lifetime AMU (%)			
	Aminoglycosides	Beta-lactams	Lincosamides	Macrolides	MLSb	Tetracyclines	Penicillins (ext.)	Lincosamides	Macrolides	Tetracyclines
Ceased parenteral and peroral tetracycline usage	-42; -4	-	-8; +0	-	-	-18; -9	-	-	-	-100
Reduction by the top 10% users of peroral tetracycline usage and replacement with peroral macrolide usage	-1; +1	+1; +3	-0; +0	+5; +7	+5; +8	-2; -1	-		24	-16
Ceased peroral and parenteral extended-spectrum penicillins usage and replacement with parenteral lincosamide usage	-	-2; -7	+13; +42	-	-	-	-100	194	-	-

In the scenario where the entire tetracycline usage were ceased, it can be expected that the tetracycline resistance abundance will be reduced by 9-18%. In addition, it can be expected that the lincosamides and aminoglycosides resistance abundance also will be reduced, even though the size of these reductions is surrounded by more uncertainty (Table 2).

In the scenario where the top 10% highest users of peroral tetracycline reduced usage by replacing it with peroral usage of macrolides, at national level, the usage of tetracyclines can be expected to be reduced by 16%, and the usage of macrolides can be expected to be increased by 24%. If the total tetracycline usage in the Danish pig production were to be reduced by app. 16%, the tetracycline resistance abundance can only be expected to be reduced by 1-2%. At the same time, the resistance abundance of beta-lactams, macrolides and MLSb can be inspected to increase relatively more compared to the reduction of tetracycline resistance abundance, due to the increased macrolide usage (Table 2).

In the scenario where parenteral and peroral usage of extended-spectrum penicillin usage were replaced with parenteral lincosamide usage, the beta-lactam resistance can be expected to be reduced by 2-7%, but the lincosamide usage and resistance abundance can be expected to be increased by 200% and 13-42%, respectively (Table 2).

Discussion

Study population

The restrictive farm selection and stratification yielded a source population representative of the vast majority of farms delivering pigs for slaughter in Denmark, which makes it possible to draw inference from the study sample to the source population. The study had an over-representation of larger farms, which was established to study the AMU-AMR associations in farms with a size and production system in alignment with the structural development in pig farms expected by the pig industry in the near future (24).

In the regression models, nothing indicated that the annual number of slaughtered pigs or the annual number of suppliers of pigs of the sampled farm interacted with the effect of lifetime AMU on the AMR abundance. Furthermore, the comparison between lifetime AMU in the finisher batches included in the observational study and all batches used for prediction, did not indicate any extrapolation of the observed results to batches with very different usage. Therefore, the batches included in the observational study can be considered representative for the investigation of the general association between lifetime AMU and AMR abundance in finishers in the Danish pig production, and the observed associations can thereby be used to predict the effect of different lifetime AMU scenarios across the pig production.

Regression analyses

Lifetime AMU was calculated as total amounts throughout the rearing period at dispensing-type and AM-class level. By doing so, it was not possible to distinguish between differences in AMU within the three rearing periods. For that reason, finisher batches with similar AMU might relate to usage in different units, thus, the AMR abundance in the gut microbiome of these pigs, may be different as changes in AMR can happen over short periods (13, 25, 26). Subsequently, the lifetime AMU does not take into account the influence of usage in the rearing period of piglets compared to usage closer to slaughter.

In the study, shotgun metagenomic sequencing was used to measure the relative AMR abundance in the microbial community of feces from finishers, therefore, the method does not distinguish between intrinsic and acquired (transferable) resistance genes in a bacterial population (27). A distinction between the two might be important for public health since intrinsic AMR poses a minor risk to humans compared to transferable AMR (27). Notwithstanding the difference between intrinsic and acquired AMR, any AMR gene in excessive abundance in habitats with high AMU, can contribute to the spread of AMR along the food chain (13). Furthermore, as the ResFinder database contains mainly AMR genes detected in clinically relevant bacteria, a considerable number of intrinsic AMR genes may have been missed (5, 13). Therefore, although not all detected AMR genes necessarily pose a risk to human health, their presence in feces from pigs represents an available gene pool from which zoonotic bacteria and human pathogenic bacteria may obtain resistance genes (12, 28, 29).

None of the significant β-coefficients in Models 1-4 of any AM-class resistances altered notably when the updated design variables or the additional AM-classes were added, indicating that the effect of AMU on AMR at AM-class level is not strongly influenced by factors as farm size, supplier number or other AMs. However, the design variables take only the finisher unit into account not the entire rearing pathway (Fig. S1). Subsequently, the updated design variables may not apply as traditional confounders for a finisher batch. Instead, characteristics such as rearing pathway or ownership through the rearing pathway could be more explanatory for finisher batches.

The significantly lower AMU and AMR abundance of most AM-classes in the organic production compared to conventional production was expected (30). The high level of beta-lactam resistance in the organic farms coincided with parenteral narrow-spectrum penicillin usage as their main drug choice. In contrast, the higher level of sulfonamide resistance in organic farms compared to conventional farm could not be explained.

The initial scatterplots of the uni-variable regression models indicated simple linear relationships between lifetime AMU and AMR abundance for all AM-classes. Hence, in the multi-variable regression models, the effects of lifetime AMU were estimated as linear. The visual inspection of the diagnostic plots of the multi-variable regression models supported the selected relationship between lifetime AMU and AMR abundance. The AMU is low in Denmark, therefore, extrapolation of the observed linear relationship between lifetime AMU and AMR abundance to higher levels of AMU should be done with caution.

Generally seen, the peroral AMs affected the AMR abundance far broader than the parenteral AMs, which in turn had a higher effect on resistance abundancy. The broader effect of peroral AMs may be due to its widespread but intermittent usage during the weaner and finisher rearing periods, which is supported by findings in other studies (31, 32). The lacking effect of parenteral extended-spectrum penicillins, macrolides and sulfonamides should be interpreted with caution, as they were foremost used at the piglet rearing period. The lifetime AMU does not distinguish between usages at different rearing periods, consequently, a usage of these AMs in the finisher unit might have had an effect on resistance abundance. Therefore, in this study it cannot be demonstrated that usage of parenteral extended-spectrum penicillins, macrolides and sulfonamides in the piglet rearing period have an effect on the resistance abundance of their corresponding AM-classes in finishers gut microbiome close to slaughter. Nonetheless, the observed difference between parenteral and peroral AMs on AMR abundance may arise from absorption, distribution and elimination of the AM substances (31, 33).

The higher effect of parenteral usage of lincosamide and tetracycline on their respective resistance abundance compared to peroral is less clear. Peroral and parenteral lincosamides were mainly used for weaners and finishers, respectively, which may explain why parenteral usage was having a higher effect on lincosamide resistance abundance. It is less clear why the effect of parenteral tetracyclines was higher than peroral tetracycline. Findings from Model 6 show that peroral tetracyclines have an effect on several genes, while the parenteral tetracyclines seemed to affect only a single gene.

The excessive effect of parenteral aminoglycosides on MLSb resistance is connected associations to three MLSb genes; *erm*(B), *erm*(F) and *erm*(G) may be due to co-selection.

The wide-ranging co-selection of macrolides in the multi-variable regression models (Model 4) was a finding with parallels to Rosengren et al. (34) which found that the occurrence of sulfamethoxazole and chloramphenicol resistance was six times higher in farms with high usage compared to farms with no usage of macrolides. In addition, co-selection of macrolide resistance by both glycopeptides and copper has previously been shown in Danish pig farms (35–37). Looft et al. also demonstrated the potential for co-selection from a single AM-class usage (38). Also, the uni-variable models of the effect of lifetime AMU on the abundance of AMR genes (Model 5) showed that peroral macrolide usage affected other AM-classes, however, primarily related to a few number of resistance genes. Similar findings were shown for the parenteral aminoglycoside usage, which affected a few number of resistance genes of several other AM-classes. The correlation matrix between abundance of AMR genes, revealed the background for the deviating behavior of *tet*(X), and the lack of effect of the sulfonamide usage on the corresponding genes

This study includes a vast amount of analyses for the uni-variable analyses at gene level and for the correlation matrix. As a consequence, it is highly probable that the false discovery rate is very high. Therefore, the significance level was reduced from 0.05 to 0.01. Despite taking this precaution, the results from these analyses should be viewed at as indicative only and as a supporting tool for the assessment and understanding of Model 4.

In general, the estimated effects of lifetime AMU on AMR abundance can be interpreted as the effect on the overall pool of AMR abundance in the gut microbiome of finishers close to slaughter in Denmark. The biological mechanism behind the observed effects is most likely that any given AM will reduce the growth of some bacteria, and thereby making room for an increase of the relative abundance of bacteria with intrinsic and acquired AMR against the given AM. This may also explain some of the unexpected effects observed, i.e. the effect that some AMs have on some of AMR abundances, even if there is no know co-resistance or cross-resistance, e.g. the effect of parenteral aminoglycoside usage on several resistance genes from different AM-classes. Although significant associations were found between AMU and AMR of every AM-class resistance, the AMU were only able to explain between 9% - 52% of the variation in AMR. While some of the un-explained variation are due to

measurement error, a substantial part still need to be explained (39).

Overall, the study has generated knowledge of the quantitative relationship between parenteral and peroral lifetime AMU and AMR abundance, at AM-class level, in the gut microbiome of finisher batches close to slaughter, which in turn can be used in predictive modelling.

Predictive model

The majority of predictive studies of occurrence of AMR has different structures in terms of complexity and inherent assumptions. Conversely, common for these models were the assumption that the treatment effect was constant (19, 40). By applying the actual information of the AMU at unit level in each farm, the movement of pigs between farms and the number of pigs delivered for slaughter from each farm, the predicted results can be anchored in the actual conditions in the Danish pig production (22).

The initial predicted AMR abundance for every batch does not include the unexplained variation in abundance between farms. Therefore, these predictions should be interpreted as a mean AMR abundance in batches with the given lifetime AMU. If the intention were to predict the AMR abundance for a specific batch, the prediction interval would be larger, because the observed unexplained variation between batches from different farms should be taken into account.

A previous validation study of VetStat data (17), demonstrated that the variation in AMR abundance that could not be explained by the lifetime AMU in finisher batches could partly be due to measurement error in the VetStat data. In addition, the validation study demonstrated that these measurement errors were also biasing the effect estimates towards zero, and the predicted effects of different lifetime AMU are therefore biased towards zero. The predictions have not been adjusted for this bias, hence, the predicted relative effects of different AMU in the pig production were conservative predictions.

The model predicted the AMR abundance given a change in the lifetime AMU within the whole or a subset of the pig production. The model has a static nature, and assumes that changes in AMR abundance are reached immediately. However, because of the living nature of the microbiome at a farm, it is expected that in reality the change will occur successively over a longer period until a new level of AMR abundance is reached.

It has proven difficult to introduce major changes in AMU that significantly reduce the occurrence of AMR in humans. A reason is that it can be very difficult to predict both exactly which interventions that will prove more efficient, i.e. how large a reduction in AMU will be needed to achieve a desired change in AMR. Previous predictive models have been based on theoretical assumptions sometimes supported by laboratory data (19, 40). This study shows that it is possible to describe the association between AMU and AMR under real-life conditions and suggest that it is feasible to develop a predictive model for a huge population, in our case the majority of pigs delivered for slaughter in Denmark, where potential scenarios can be tested. In itself, this provides a significant tool for the Danish authorities and other stakeholders, but it also provides an example for what is feasible and which data will be needed in order to provide guidance for major political and targeted interventions in production animals and humans globally. Thus, this study provides a framework for further development, which might eventually assist in reducing AMR and safeguard AMs for the future.

Methods

Study design

The study was designed as an observational cross-sectional study of Danish pig farms that delivers more than 800 pigs annually and received pigs from a maximum of four supplier annually. The selected farms were stratified, based on the farm characteristics; production-type (conventional or organic), annual number of pigs delivered for slaughter and annual number of suppliers of pigs. The aim of the stratification was to obtain a representative study sample compared to farms producing the majority of pigs slaughtered annually in Denmark, with the predominant supplier paths.

Based on explorative analyses of data from 2013 from the Central Husbandry Register (CHR), conventional farms with >200 pen-places for finishers accounted for the delivery of more than 98% of the finishers slaughtered in Denmark. The corresponding percentage of organic farms was 95% (Table 3). On average, 200 pen-places for finishers are the equivalent to delivering app. 800 pigs for slaughter annually. Based on data from 2013 from the Pig Movement Database (PMD), farms delivering more than 800 pigs for slaughter annually were found. Among these, farms were selected using a maximum of four as cut off value for annual number of suppliers (Table 3). Subsequently, of the 19.3 million pigs slaughtered that year, the selected source population comprised 90% of the total number of slaughtered pigs (41), and 57% of the total number of pig farms (Table S3).

Then the source population was separated into two sub-populations; i) the current most common farm size delivering pigs to the pork industry in Denmark and ii) larger scale farms delivering more than 5000 slaughtered pigs annually, which is expected to be the predominant farm size to deliver slaughter pigs in the near future (42). Afterwards, the two sub-populations were separated based on the annual number of suppliers, purposely to signify ownership complexity compared to the rearing pathway of finisher batches. Thus, a rearing pathway with 0-1 supplier annually was assumed farms, owned by one farmer, and a rearing pathway with 2-4 suppliers was assumed farms owned by different farmers. The source population included 3,859 conventional and 24 organic pig farms, which were stratified into four groups based on the annual number of suppliers (0-1 supplier / 2-4 suppliers of pigs per year) and the annual number of pigs sent for slaughter (800-4999/ ≥5000 slaughtered pigs per year). For purpose of comparison, the organic farms were chosen in Group 1 only (Table 3).

Identification of study sample

The collection and laboratory analysis of samples from about 80 farms aligned with the overall resources in the project, thereby aiming for app. 20 farms in each group – 80 conventional and 5 organic.

The list of potential farms was randomized within each stratum, and initially, letters of invitation were sent to 20 farms in each group. Farms were then contacted by telephone in the following weeks to determine if they were interested in participating and if so, to plan the visits. Hereafter, 20 additional farms across the groups were invited. Farms were invited and contacted in groups of 10-20 farms at a time until 83 farms had agreed to participate; 78 conventional and 5 organic. In total, eight rounds of invitation-letters were sent. Two thirds of the invited farmers agreed to participate, thus, for the strata 1 to 4; 23, 13, 22 and 25, respectively, participated in the study. Five of the 23 farms in stratum 1 where farms with organic production. Subsequently, 83 farms stratified into four groups were included in the study sample (Table 3).

Table 3. The stratified distribution of number of farms and slaughtered pigs in the source population in 2013 in Denmark and number of farms in the study sample of conventional and organic production.

Strata	Source population	Study sample

		Farms		Slaught.†		Farms	
Suppl.*	Slaught.†	Conv.‡	Org.§	Conv.‡	Org.§	Conv.‡	Org.§
0 -1	800-4999	1,816	24	4,773,000	54,921	18	5
2 - 4	800-4999	685	-	1,835,966	-	13	-
0 -1	≥5000	938	-	7,398,554	-	25	-
2 - 4	≥5000	420	-	3,325,021	-	22	-
Total		3,859	24	17,332,541	54,921	78	5

* Annual number of suppliers of pigs
† Annual number of slaughtered finishers
‡ Conventional production
§ Organic production

From each farm, samples were collected from the oldest finishers. With the help of the farmer, all sections containing finishers >80kgs were identified. The number of pens was counted and randomized using random.org/lists. Hereafter the 30 first pens on the list were sampled. In case the farm had less than 30 pens containing finishers >80kg, each pen was sampled 2-3 times in order for the total number of samples to reach 24-30 pens. One person along with an assistant carried out the sampling in all farms. The sampling material was fecal material collected directly from the pigs as they defecated or immediately after from the pen floor. If collected from the floor, only the top of an undisturbed pile was collected. In the laboratory, each sample was mixed thoroughly and an equal amount from each sample was weighed out and pooled into one. This composite sample was then used for further analyses. Sampling took place from December 2014 to Marts 2016.
For transportation to the laboratory, the samples were placed in a thick walled polystyrene box along with cooling elements. Within 6 and 24 hours of collection, the samples were placed in 4°C storage and were processed in the laboratory, respectively.

Data sources
Data on AMU was obtained from the Danish Veterinary Medicine Statistic Program database (VetStat), which contains records on purchased medicines prescribed by veterinarians for animals. Each record has information on the product name, substance, dispensing-type, amount, target species, age-group, diagnosis group and farm code (ID) (43). Data from VetStat were extracted two years before and three months after the sampling date of each farm to establish sufficient buffer time before and after the study periods to account for negative entries (44). The data were then cleaned according to guidelines by correcting mismatches of animal species and/or age-group by cross-validating the data with CHR data (44).
In order to produce comparable data across records, substances were converted into a unit measuring how many kilograms of pig could be treated per day, known as – Animal Defined Daily Doses per kilogram (*ADD (mg/kg)*) (45).
Data on farms were obtained from the CHR, which stores information linked to a farm code (ID) referring to a specific geographical location, and data on movements of pigs were obtained from the PMD, which records the number of pigs, date, ID of origin farm and ID of destination farm for each movement (43, 46). By combining data from the CHR and PMD, the movements of pigs between farms and the annual number of pigs moved out of a farm either to another farm or to a slaughterhouse could be obtained (43, 46). As adjustment factor for farm size, a proxy measure was calculated. First, the annual production of sold and/or slaughtered pigs in a farm were multiplied by the national productivity averages for piglets, weaners and finishers. Thus, the number of days to produce a piglet, weaner and finisher, 30, 55, and 85 days, respectively, which then were divided by 365 days in order to calculate the number of piglets, weaners and/or finisher on any given day in a farm (41).

Estimation of daily AMU
The AMU was calculated for all AM product records *(l)*, during a period *(k)*, in an age-group *(j)* (piglets /weaners/finishers) in a farm *(i)* as $ADDkg_{ijkl,,}$ measured as *ADDkg/pig.day* using the formula:

$$ADDkg_{ijkl}\ (ADDkg/pig.day) = \frac{product_{ijkl}\ (mg)}{days_k * ADD_l\ (mg/kg) * pigs_{ijk}}$$

where: *product* = the number of milligram of an AM product in a specific farm/age-group/period, *days* = the interval in days between the day of the initial VetStat record and the day of the subsequent record, *ADD* = standard doses, and *pigs* = the number of piglets/weaners/finishers on any given day in a farm (16, 17).
To calculate $ADDkg_{ijkl,}$ based on VetStat records, the *days* was estimated for peroral as; *days* between records at farm, age-group and dispensing-type level, and for parenteral as; *days* between records at farm, age-group, dispensing-type and AM-class level (17). In addition, the calculation of *days* was based on three assumptions. First, if the number of *days* was less than eight, the following subsequent record date was used instead. Second, if no subsequent date was found, the mean of the former intervals in days was applied. If no prior number of *days* was available, 90 and 365 was utilised for peroral and parenteral dispensing-type, respectively. Third, all numbers of *days* exceeding 90 days for peroral dispensing and 365 days for parenteral dispensing-type were replaced with 90 and 365 days, respectively (17).

Estimation of lifetime AMU
The lifetime AMU is an average estimate that related to usage during the entire rearing period independent of rearing site (16). The 83 finisher batches pathways were established by following them through rearing sites from the sampling farm back to farm of birth. Danish national averages for pig production productivity for 2015 were applied for the rearing periods per unit (in days) (14), resulting in 30, 55 and 85 days in the farrowing (piglet), weaning (weaner) and finisher units (finisher), respectively (Fig. S1).
Then the AMU for the 83 finisher batches, was calculated as the sum of the date- and product-specific AMU ($ADDkg_{ijkl}$) for each AM-class at dispensing-type level over the rearing periods; piglet, weaner and finisher. Next, the obtained amount was adjusted to suit the proportion of animals being moved from a farm. Finally, the absolute lifetime AMU for each AM-class at dispensing-type level were calculated for each finisher batch by summarising the AMU in the three units, given by their rearing pathways (Fig. S1). The lifetime AMU quantifies the total number of kg-doses per pig during the rearing period of 170 days. Even though AMU for sows was included in the usage for piglets, previous studies have shown that this affects the occurrence of AMR in the gut microbiome of piglets, thus, it was assumed that the usage of sows was equivalent to the usage of piglets (47).

Estimation of AMR
The methods used to extract DNA and obtain AMR abundance in the microbiome of finisher batches have been described in previous studies (13, 48). In brief, genes encoding resistance to aminoglycosides, beta-lactams, lincosamides, macrolides, macrolides-lincosamide-streptogramin B (MLSb), sulfonamides and tetracyclines, were quantified using shotgun metagenomic sequencing, and measured as Fragments per Kilobase reference per Million fragments (*FPKM*) (13).

DNA extraction
For DNA extraction, a modified QIAamp Fast DNA Stool Mini Kit protocol was employed Qiagen, Valencia, CA, USA). Cryotubes with pooled faeces were gently thawed on ice. Prior to the protocol, 0.2 g of sample was mixed with 1 mL of InhibitEX buffer in a Lysing Matrix A tube (MP Biomedicals). Samples were treated with a TissueLyser (3×30 s, 30 Hz) and were chilled on ice between

repetitions. Following bead beating, samples were heated to 95°C for 7 min and centrifuged to eliminate larger stool particles. Then. DNA was eluted in 100 mL of elution buffer (13, 48).

DNA sequencing
PCR-free DNA libraries were generated and sequenced on the HiSeq2500 (Illumina) to generate roughly 7 gigabases of paired-end reads per sample, enough to get 20×coverage of bacteria with 1% abundance (13, 48).

Read mapping (resistance)
Resistance was quantified using the MGmapper tool against the ResFinder database, April 2017 (49). The database contains several highly homologous genes, thus, when reads map to identical parts of homologous gene variants unspecific mapping occurs. Read counts from variants of the same gene were aggregated to gene levels according to common gene names, resulting in the final abundance matrix of 135 genes for the 83 finisher batches.

Metagenomic data analysis
For each AM-class, the raw read counts were normalized to length of each gene and sequencing depth of each sample, thus, measured as Fragments per Kilobase reference per Million fragments (*FPKM*) using the formula:

$$FPKM = \frac{\left({}^{n}/_{(N * (l - (i - 2 * m))}\right)}{2 * 10^{6} R * 1000 bp}$$

, where n = number of mapped reads, N = total number of reads, l = gene length, i = insert size, m = minimum mapping length, R = Reads and bp = base pair.
The normalization takes into account that the pooling and sequencing of several indexed samples produces varying DNA library sizes, resulting in comparable *FPKM* values that are independence of sequencing depth.

Data analyses
The data analyses started by focusing on the quantitative usage of an AM-class and abundance of the respective AM-class resistance. Subsequently, uni-variable regression models were used to calculate the effect of lifetime AMU on the AMR abundance for the AM-classes; aminoglycosides, extended-spectrum penicillins vs. beta-lactams, lincosamides, macrolides, macrolides (MLSb), sulfonamides, and tetracyclines. When modelling, spectinomycin, and trimethoprim were not included in aminoglycosides and sulfonamides, respectively, instead, they constituted separate AM-classes in the multi-variable models (Models 4-5) (Fig. 3). Focusing on the biologically most plausible AMU-AMR relationships in the initial analyses, will prevent overemphasizing results from more complex regression analyses that estimate the effect of several AM-classes simultaneously. However, by including usage of several AM-classes, the potential effects related to co-resistance and cross-resistance were estimated. The diagnostic plots were applied for visual inspection of all of the estimated models, in order to assess if the assumptions of linear regression were fulfilled. The visual inspection included; whether the residuals were normally distributed and showed homoscedasticity. The assessment of potential influential observations was done using Cook's distance (>1). Furthermore, all models were assessed for the influence of potential outlying observations on the estimated effects using bi-square robust regression (50).

Regression analyses
The analytical work was performed in several steps (Fig. 3):

1. Uni-variable model - Model 1

First, lifetime usage of aminoglycosides, extended-spectrum and narrow-spectrum penicillins, lincosamides, macrolides, sulfonamides and tetracyclines were plotted against aminoglycoside, beta-lactam, lincosamide, macrolide and MLSb, sulfonamides and tetracycline resistance abundance, respectively, in scatterplots. Hereafter, a LOESS local regression model using span width 0.75 was added to each scatterplot to evaluate the relationship between lifetime AMU and AMR abundance (51). Based on visual inspections of the LOESS regression lines, it was decided to model the effect of AMU on AMR as linear. Afterwards, the estimated linear regression models with 95% CI of the respective AM-class were overlaid the observations in the scatterplots. The scatterplots were finalized by implementing bi-square robust regression.

2. Model 1 included updated design variables - Model 2

To adjust the estimates for potential confounding, the updated design variables; production-type, number of slaughtered pigs and suppliers of pigs per year were added to the uni-variable models (Model 1). First, production-type was included to adjust for confounding, due to the uneven number between conventional and organic farms in the study sample. Subsequently, as a result of the organic farms significant effect in all models, the remaining regression analyses were performed for the conventional farms only. The variables, number of slaughtered pigs and number of suppliers of pigs per year, were first included one at a time, and afterwards, simultaneously to adjust for confounding.

3. Multi-variable model at dispensing-type level - Model 3

In model 3, the effect of lifetime AMU at dispensing-type level of aminoglycosides, extended-spectrum penicillins, lincosamides, macrolides, sulfonamides and tetracycline on the AMR abundance of aminoglycosides, lincosamides, macrolides/MLSb, sulfonamides and tetracyclines were estimated, thereby estimating the effect of peroral and parenteral lifetime AMU on the respective AM-class resistance. The β-coefficients of parenteral and peroral dispensing-type of the AM-class specific variable(s) were then compared to the β-coefficients of the initial uni-variable models (Model 1) to assess the difference between them.

4. Multi-variable model included all AM-classes at dispensing-type level and updated design variables - Model 4

Then, multi-variable regression models included the potential direct and confounding effects of use of AM-classes other than the AM-class specific as well as updated design variables were performed. These models included the effect of parenteral and peroral lifetime AMU of aminoglycosides, extended-spectrum and narrow-spectrum penicillins, lincosamides, macrolides, pleuromutilins, polymyxins, spectinomycin, sulfonamides, tetracyclines and trimethoprim on abundance of resistance of aminoglycosides, beta-lactams, lincosamides, macrolides, MLSb, sulfonamides and tetracyclines. The models were obtained using automated stepwise regression, with the AIC value as the criteria for variable selection (50, 51). Insignificant variables in the models were removed using manual stepwise exclusion. The comparison between models were performed using the ANOVA; Chi-square test. When these were not significantly different (p-value > 0.05), the simpler model was preferred.
The explanatory variables kept in the models were checked for potential correlation. Afterward, the β-coefficients of the AM-class variable(s) specific for the AM-class resistance were compared to the β-coefficients of the multi-variable model-variable models (Model 3) to assess the difference between them. The final models were assessed for the influence of potential outlying observations on the estimated effects with bi-square robust regression.

5. Uni-variable model at resistance gene level – Model 5

At the gene level, uni-variable regression models of the effect of each lifetime AMU of the AM-classes; aminoglycosides, extended-spectrum and narrow-spectrum penicillins, lincosamides, macrolides, pleuromutilins, polymyxins, spectinomycin, sulfonamides, tetracyclines and trimethoprim at parenteral and peroral level, on abundance of each resistance gene of aminoglycosides, beta-lactams, lincosamides, macrolides, MLSb, sulfonamides and tetracyclines were estimated. Genes that were found in less than 10 samples and regression results with p-values above 0.01 were not included in the assessment.

6. Correlation matrix at resistance gene level - Matrix 6

Based on the genes of the AM-classes; aminoglycosides, beta-lactams, lincosamides, macrolides, MLSb, sulfonamides and tetracyclines, a correlation matrix using the spearman method was estimated (52). Genes that were found in less than 10 samples and correlation coefficient results with p-values less than 0.01 were excluded from assessment.

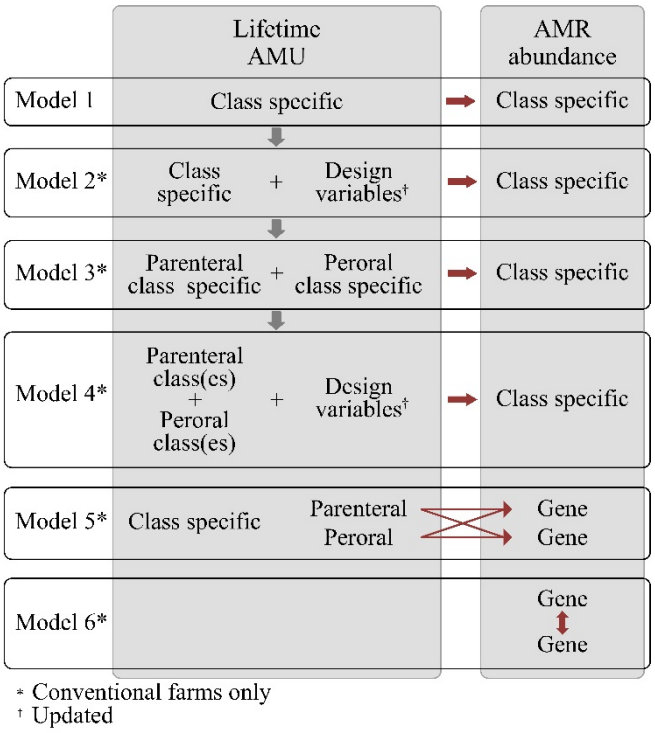


Fig. 3. Overview of the regression analyses (Model 1-5) and correlation analysis (Model 6) performed.

Predictive model

The estimated effects of AMU on AMR in the presented observational study, the easy accessibility to data on AMU at pig farms (VetStat) and movements of the vast majority of pigs between farms (PMD) in Denmark, provide the opportunity to predict the overall AMR abundance in the majority of Danish finishers close to slaughter at national level (22, 23). Consequently, this framework also makes it possible to predict the relative effect of different AMU in all or a subset of pig farms on the overall AMR abundance in Danish finishers close to slaughter. In these predictions, it is assumed that the estimated effects of lifetime AMU on AMR abundance can be generalized across conventional pigs produced and slaughtered in Denmark.

As an input to the simulation, the lifetime AMU in finisher batches delivered from finisher farms to slaughterhouses in Denmark was estimated based on data of finisher batches delivered for slaughter from April 2014 to June 2014. In this period, 3,079 finisher batches were delivered for slaughter. The lifetime AMU in finishers in each batch was estimated using a previously described algorithm (22).

Initially in the simulation, the mean AMR abundance in finishers of each of the 3,079 batches was predicted using the estimated regression models (Model 4) from the observational study. Secondly, to obtain the abundance at national level, the number of finishers delivered for slaughter during the last 6 months from the corresponding farm was multiplied by the estimated mean of AMR abundance in a batch. As a result, the AMR abundance related to the different AM-classes was summarized across all batches and constitute the baseline measure of AMR abundance.

Simultaneously, the same calculation was performed using data of AMU mimicking several different AMUs in either all of the 3,079 batches or in a subset of those as input. The relative effect on the AMR abundance of the different AMUs scenarios were obtained by comparing the abundance of AMR to the baseline abundance.

To include the uncertainty in the estimated regression parameters, the above calculation of AMR abundance and the relative change was carried out 100,000 times (iterations), and in each iteration, random values of the effect parameters were selected from Gaussian probability distributions. These distributions were defined using the point estimate (β-coefficient) of the effect as the mean and the standard error of the point estimate as standard deviation. Thereby, creating an uncertainty distribution around the relative effect of different lifetime AMU, which expressed the uncertainties in the estimated effect parameters. To avoid simulation noise in the confidence intervals, the same values for the effect parameters were used in the baseline and the different scenarios within each iteration. The validity of generalizing the estimated models to the overall production in Denmark was assessed by comparing the "location" of the lifetime AMU in the finisher batches included in the observational study across the deciles of the lifetime AMU in all finisher batches used in the prediction.

The prediction model presents results from three different scenarios of lifetime AMU in the Danish pig production. In the first scenario, all parenteral and peroral tetracycline usage was ceased without replacement. In the second scenario, the reduction of the top-10% farms of peroral usage of tetracyclines per produced pig to the level just below these, combined with an equivalent replacement in doses of lifetime peroral macrolide usage. In the third scenario, all parenteral and peroral usage of extended-spectrum penicillins was ceased, combined with an equivalent replacement in doses of lifetime parenteral lincosamide usage.

WPS Workbench, Version: 3.1.1.0.0, Microsoft Excel 2010, and R, version 3.3.3 were applied in all data processing and data analyses. The predictive modelling was performed using @RISK – risk analysis Add-in for Microsoft Excel, version 7.5.1.

Acknowledgments

We thank all the farmers for participating in this study. We thank our colleagues from the National Food Institute of the Technical University of Denmark for their dedicated technical assistance with DNA extraction.

Funding

This work was supported by the Danish Ministry of Food, Agriculture and Fisheries.

Conflicts of interest

The authors declare no conflict of interest

Author contributions: H.V., F.M.A., L.V.d.K., and V.D.A. designed research; H.V., F.M.A., P.M, M.S.J., L.V.d.K., B.E.K., O.L., V.B., A.C.B., and V.D.A. performed research; H.V., P.M, L.V.d.K., O.L., and V.D.A. analyzed data; and H.V., F.M.A., P.M, M.S.J., L.V.d.K., B.E.K., O.L., V.B., A.C.B., and V.D.A. wrote the paper.

References

1. WHO (2012) The evolving threat of antimicrobial resistance - Options for action (World Health Organization) Available at: http://www.who.int/patientsafety/implementation/amr/publication/en/index.html.
2. Friedman ND, Temkin T, Carmeli Y (2016) The negative impact of antibiotic resistance. Clin Microbiol Infect 22(5):416–422.
3. Jensen HH, Hayes DJ (2014) Impact of Denmark's ban on antimicrobials for growth promotion. Curr Opin Microbiol 19(1):30–36.
4. Tang KL, et al. (2017) Restricting the use of antibiotics in food-

producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. Lancet Planet Heal 1(8):e316–e327.

5. Marshall BM, Levy SB (2011) Food animals and antimicrobials: Impacts on human health. Clin Microbiol Rev 24(4):718–733.

6. Aarestrup FM (2015) The livestock reservoir for antimicrobial resistance: a personal view on changing patterns of risks, effects of interventions and the way forward. Philos Trans R Soc Lond B Biol Sci 370(1670). doi:10.1098/rstb.2014.0085.

7. Holmes AH, et al. (2016) Understanding the mechanisms and drivers of antimicrobial resistance. Lancet 387(10014):176–187.

8. European Medicines Agency. European Surveillance of Veterinary Antimicrobial Consumption (2016) Sales of veterinary antimicrobial agents in 29 European countries in 2014 Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2016/10/WC500214217.pdf.

9. Wielinga PR, Jensen VF, Aarestrup FM, Schlundt J (2014) Evidence-based policy for controlling antimicrobial resistance in the food chain in Denmark. Food Control 40(1):185–192.

10. Aarestrup FM, et al. (2001) Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. Antimicrob Agents Chemother 45(7):2054–2059.

11. Agersø¸ Y, Aarestrup FM (2013) Voluntary ban on cephalosporin use in Danish pig production has effectively reduced extended-spectrum cephalosporinase-producing Escherichia coli in slaughter pigs. J Antimicrob Chemother 68(3):20569–20572.

12. Collineau L, et al. (2017) Guidance on the Selection of Appropriate Indicators for Quantification of Antimicrobial Usage in Humans and Animals. Zoonoses Public Health 64(3):165–184.

13. Munk P, et al. (2017) A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds. J Antimicrob Chemother 72(2). doi:10.1093/jac/dkw415.

14. SEGES (2016) ANNUAL REPORT 2015 Available at: http://eng.vsp.lf.dk/~/media/Files/PDF - Aarsberetning VSP English/VSPårsberetning UK 2015.pdf.

15. DANMAP (2015) DANMAP – DADD Available at: hhtp://www.danmap.org.

16. Andersen VD, et al. (2017) The association between measurements of antimicrobial use and resistance in the faeces microbiota of finisher batches. Epidemiol Infect:1–11.

17. Andersen VD, et al. Validation of the antimicrobial lifetime exposure measurement for finisher batches based on comparison to registered antimicrobial usage at farm level. Epidemiol Infect.

18. Abatih EN, Alban L, Ersbøll AK, Lo Fo Wong DM (2009) Impact of antimicrobial usage on the transmission dynamics of antimicrobial resistant bacteria among pigs. J Theor Biol 256(4):561–573.

19. Spicknall IH, Foxman B, Marrs CF, Eisenberg JN (2013) A modeling framework for the evolution and spread of antibiotic resistance: literature review and model categorization. Am J Epidemiol 178(4):508–520.

20. Arepyeva M, Kolbin A, Kurylev A, Balykina J, Sidorenko S (2015) What should be considered if you decide to build your own mathematical model for predicting the development of bacterial resistance? Recommendations based on a systematic review of the literature. Front Microbiol 6(APR):352.

21. Arepyeva MA, et al. (2017) A mathematical model for predicting the development of bacterial resistance based on the relationship between the level of antimicrobial resistance and the volume of antibiotic consumption. J Glob Antimicrob Resist 8:148–156.

22. Birkegård AC, et al. (2017) Computional algorithm for lifetime exposure to antimicrobials in pigs using register data—The LEA algorithm. Prev Vet Med 146:173–180.

23. Birkegård AC, et al. (2017) Association between selected antimicrobial resistance genes and antimicrobial exposure in Danish pig farms. Sci Rep 7(1). doi:10.1038/s41598-017-10092-9.

24. Christiansen MG (2014) No 1429: The Structural Development in the Danish Pig Production 2014 [in Danish]. SEGES:11.

25. Dawson KA, Langlois BE, Stahly TS, Cromwell GL (1984) Antibiotic resistance in anaerobic and coliform bacteria from the intestinal tract of swine fed therapeutic and subtherapeutic concentrations of chlortetracycline. J Anim Sci 58(1):123–131.

26. Cavaco LM, Abatih E, Aarestrup FM, Guardabassi L (2008) Selection and persistence of CTX-M-producing Escherichia coli in the intestinal flora of pigs treated with amoxicillin, ceftiofur, or cefquinome. Antimicrob Agents Chemother 52(10):3612–3616.

27. Martinez JL, Coque TM, Baquero F (2015) What is a resistance gene? Ranking risk in resistomes. Nat Rev Microbiol 13(2):116–123.

28. SCENIHR (2009) Assessment of the Antibiotic Resistance Effects of Biocides (European Commission) Available at: http://ec.europa.eu/health/archive/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf.

29. Cavaco LM, Hasman H, Aarestrup FM (2011) Zinc resistance of Staphylococcus aureus of animal origin is strongly associated with methicillin resistance. Vet Microbiol 150(3–4):344–348.

30. Österberg J, et al. (2016) Antibiotic Resistance in Escherichia coli from Pigs in Organic and Conventional Farming in Four European Countries. PLoS One 11(6):e0157049.

31. Wiuff C, Lykkesfeldt J, Svendsen O, Aarestrup FM (2003) The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of quinolone resistance among Salmonella and coliforms in pigs. Res Vet Sci 75(3):185–193.

32. Zhang L, Huang Y, Zhou Y, Buckley T, Wang HH (2013) Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota. Antimicrob Agents Chemother 57(8):3659–66.

33. Bibbal D, et al. (2007) Impact of three ampicillin dosage regimens on selection of ampicillin resistance in Enterobacteriaceae and excretion of blaTEM genes in swine feces. Appl Environ Microbiol 73(15):4785–4790.

34. Rosengren LB, Waldner CL, Reid-Smith RJ, Dowling PM, Harding JCS (2007) Associations between feed and water antimicrobial use in farrow-to-finish swine herds and antimicrobial resistance of fecal Escherichia coli from grow-finish pigs. Microb Drug Resist 13(4):261–270.

35. Aarestrup FM (2000) Characterization of glycopeptide-resistant Enterococcus faecium (GRE) from broilers and pigs in Denmark: Genetic evidence that persistence of GRE in pig herds is associated with coselection by resistance to macrolides. J Clin Microbiol 38(7):2774–2777.

36. Hasman H, Aarestrup FM (2005) Relationship between copper, glycopeptide, and macrolide resistance among Enterococcus faecium strains isolated from pigs in Denmark between 1997 and 2003. Antimicrob Agents Chemother 49(1):454–456.

37. Hasman H, et al. (2006) Copper resistance in Enterococcus faecium, mediated by the tcrB gene, is selected by supplementation of pig feed with copper sulfate. Appl Environ Microbiol 72(9):5784–5789.

38. Looft T, et al. (2012) In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci U S A 109(5):1691–1696.

39. Andersen VD, et al. (2018) Validation of the register-based lifetime antimicrobial usage measurement for finisher batches based on comparison with recorded antimicrobial usage at farm level. Epidemiol Infect:1–9.

40. Blanquart F, Lehtinen S, Fraser C (2017) An evolutionary model to predict the frequency of antibiotic resistance under seasonal antibiotic use , and an application to Streptococcus pneumoniae Franc. Proc R Soc. doi:dx.doi.org/10.1098/rspb.2017.0679.

41. SEGES (2015) ANNUAL REPORT 2014 Available at:

http://eng.vsp.lf.dk/~/media/Files/PDF - Aarsberetning VSP English/Årsberetning 2014_UK.pdf.

42. Jensen T, et al. (2011) Assessment of the future production systems for pigs [in Danish only. Summary available in English] Available at: http://vsp.lf.dk/Publikationer/Kilder/lu_Rapporter/38.aspx.

43. Stege H, Bager F, Jacobsen E, Thougaard A (2003) VETSTAT-the Danish system for surveillance of the veterinary use of drugs for production animals. Prev Vet Med 57(3):105–115.

44. Dupont N, et al. (2017) Improving institutional memory on challenges and methods for estimation of pig herd antimicrobial exposure based on data from the Danish Veterinary Medicines Statistics Program (VetStat). Available at: http://arxiv.org/abs/1705.08663.

45. Jensen VF, Jacobsen E, Bager F (2004) Veterinary antimicrobial-usage statistics based on standardized measures of dosage. Prev Vet Med 64(2–4):201–215.

46. Houe H, Gardner IA, Nielsen LR (2011) Use of information on disease diagnoses from databases for animal health economic, welfare and food safety purposes: strengths and limitations of recordings. Acta Vet Scand 53(suppl 1):S7.

47. Callens B, et al. (2015) Presence of Antimicrobial Resistance and Antimicrobial Use in Sows Are Risk Factors for Antimicrobial Resistance in Their Offspring. Microb Drug Resist 21(1):50–58.

48. Knudsen BE, et al. (2016) Impact of Sample Type and DNA Isolation Procedure on Genomic Inference of Microbiome Composition. mSystems 1(5):e00095-16.

49. Zankari E, et al. (2012) Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67(11):2640–2644.

50. Venables WN, Ripley BD (2002) Modern Applied Statistics with S Fourth edition by doi:10.2307/2685660.

51. Hadley W (2017) Easily Install and Load “Tidyverse” Packages. Available at: https://www.tidyverse.org/.

52. Wei T, Simko V (2017) R package “corrplot”: Visualization of a Correlation Matrix (Version 0.84). Available at: https://github.com/taiyun/corrplot

5.2 Supplementary material

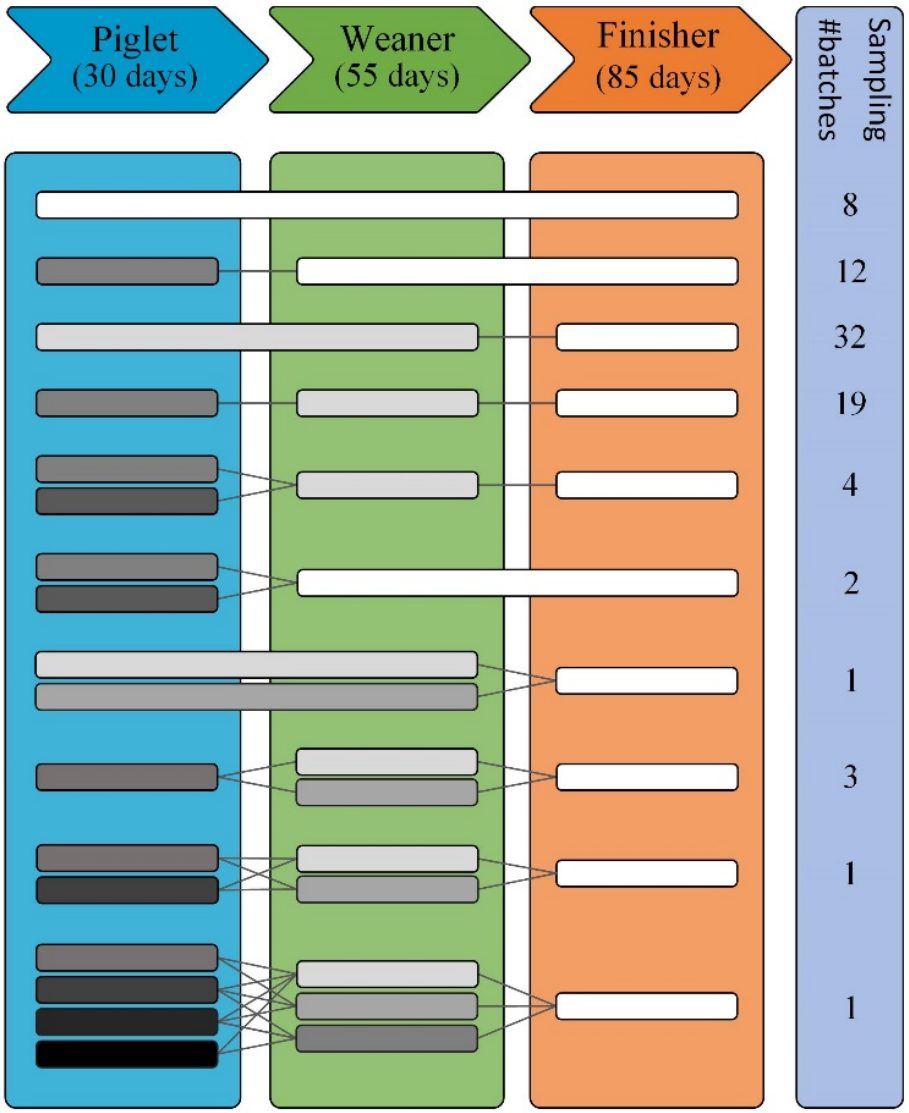


Fig. 5.2.1. The rearing pathways of the 83 finisher batches from birth site to finisher site compared with the day of sampling. From left, the first three vertical coloured bars represent the assumed days of antimicrobial usage (AMU) in the; sow-piglet (farrowing) unit, weaner unit and finisher unit. Each rectangle within the vertical bars represents a farm at a specific geographical location. Therefore, the rectangular size of a bar and a colour shift in a bar denotes that a farm has a different geographical location compared with the farm where sampling took place. The 10 different horizontally coherent bars depict the different rearing pathways of the 83 finisher batches. The fourth vertical bar shows, the number of sampled finisher batches per horizontal coherent bar.

Fig. 5.2.2. The parenteral and peroral lifetime AMU, measured as *ADDkg/pig* of the AM-classes; aminoglycosides including spectinomycin, lincosamides, extended-spectrum penicillins, pleuromutilins, polymyxins, macrolides, narrow-spectrum penicillins, sulfonamides including trimethoprim and tetracyclines of the 83 finisher batches, ranked according to the total lifetime AMU in the batches.

Table 5.2.3. The coefficients with 95% confidence interval (CI), standard error (SE) and p-values of the multi-variable linear regression models (Model 4) of parenteral and peroral lifetime AMU of all AM-classes on each AM-class and updated design variables on resistance abundance of aminoglycosides, beta-lactams, lincosamides, macrolides, MLSb, sulfonamides and tetracyclines, including the coefficients of bi-square robust regression analyses (rlm). Furthermore, for all models, the statistical estimate of model fit; adjusted R-squared (Adj R^2), was applied.

Resistance	coef-ficients	coefficients (95% CI)	SE	p-value	Adj. R^2	rlm coefficients
Aminoglycosides						
Model 4					0.25210	
(intercept)	6.33825	(4.79942 - 7.87709)	0.77194	0.00000 ***		6.19630
Aminoglycosides (parenteral)	0.34091	(0.14388 - 0.53794)	0.09884	0.00094 ***		0.36440
Macrolides (peroral)	0.00943	(0.00096 - 0.01790)	0.00425	0.02969 *		0.00930
Pleuromutilins (peroral)	0.01715	(0.00574 - 0.02856)	0.00572	0.00375 **		0.01180
Tetracyclines (parenteral)	0.03758	(0.00263 - 0.07253)	0.01753	0.03544 *		0.03910
Tetracyclines (peroral)	0.01177	(-0.00003 - 0.02357)	0,01177	0.05055 .		0.01290
Beta-lactams						
Model 4					0.15150	
(intercept)	29.67841	(26.60727 - 32.74901)	1.54152	0.00000 ***		29.01510
Extended-spectrum penicillins (peroral)	0.15211	(0.05605 - 0.24818)	0.04822	0.00231 **		0.15100
Macrolides (peroral)	0.03464	(0.00823 - 0.06106)	0.01326	0.01086 *		0.03720
Lincosamides						
Model 4					0.24280	
(intercept)	26.53758	(23.17420 - 29.90276)	1.68889	0.00000 ***		26.36700
Lincosamides (parenteral)	0.59117	(0.20080 - 0.98155)	0.19592	0.00349 **		0.62970
Lincosamides (peroral)	0.32771	(0.16425 - 0.49117)	0.08203	0.00015 ***		0.33000
Tetracyclines (parenteral)	0.11352	(0.00312 - 0.22391)	0.0554	0.04402 *		0.07280
Macrolides						
Model 4					0.44180	
(intercept)	53.64111	(45.84518 - 61.43703)	3.91426	0.00000 ***		53.20090
Macrolides (peroral)	0.28225	(0.21083 - 0.353579)	0.03586	0.00000 ***		0.28620
MLSb						
Model 4					0.52380	
(intercept)	15.63985	(12.52425 - 18.75546)	1.56398	0.00000 ***		13.65330
Aminoglycosides (parenteral)	0.81576	(0.16757 - 1.46394)	0.32538	0.01430 *		0.83680
Macrolides (peroral)	0.10649	(0.07923 - 0.13375)	0.01368	0.00005 ***		0.11350
Sulfonamides						

Resistance	coef-ficients	coefficients (95% CI)		SE	p-value	Adj. R^2	rlm coefficients
Model 4						0.08876	
(intercept)	0.09696	(0.05936 -	0.13456)	0.01888	0.00000 ***		0.06810
Polymyxins (peroral)	0.00343	(0.00109 -	0.00577)	0.00118	0.00466 **		0.00400
Tetracyclines							
Model 4						0.26780	
(intercept)	266.70323	(247.08080 -	286.32567)	9.84793	0.00000 ***		266.63440
Macrolides (peroral)	0.13280	(0.01331 -	0.25229)	0.05997	0.02990 *		0.11960
Tetracyclines (parenteral)	0.67628	(0.15015 -	1.20241)	0.26405	0.01250 *		0.67080
Tetracyclines (peroral)	0.44507	(0.26758 -	0.62255)	0.08907	0.00000 ***		0.45440

* Level of significance (0: ***, 0.001: **, 0.01: *, 0.05: .)

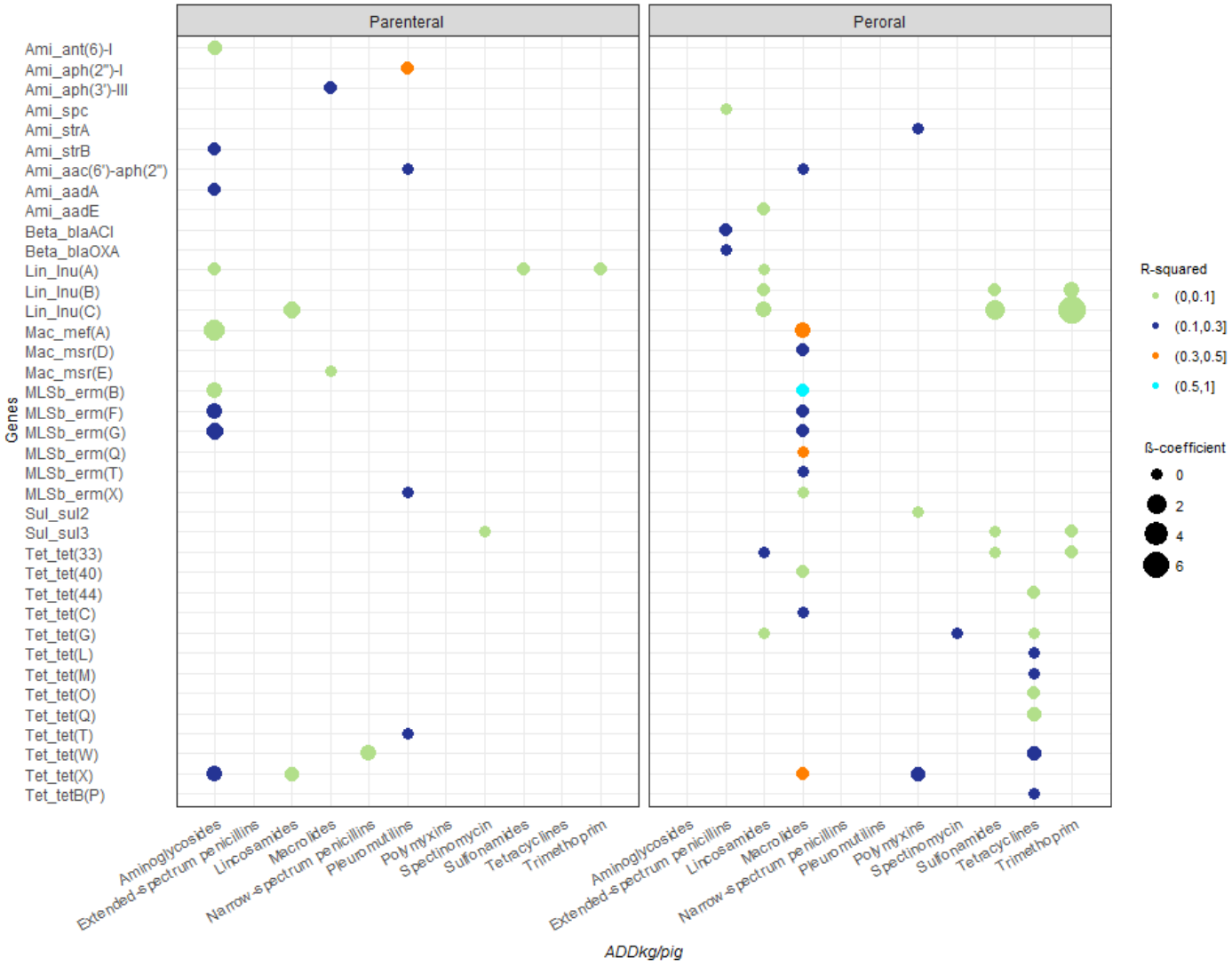


Fig. 5.2.4. The β-coefficients from the uni-variable regression analyses of parenteral and peroral lifetime AMU of aminoglycosides, extended-spectrum penicillins, lincosamides, macrolides, narrow-spectrum penicillins, pleuromutilins, polymyxins, spectinomycin, sulfonamides, tetracyclines and trimethoprim on the abundance of all resistance genes within the AM-classes; aminoglycosides, beta-lactams, lincosamides, macrolides, MLSb, sulfonamides and tetracyclines. Only results from linear regression analyses with p-values of less than 0.01 are plotted. The size of a point illustrates the size of the β-coefficient. The colour of a point, illustrates the variation of the resistance genes that the antimicrobial usage was able to explain (R^2).

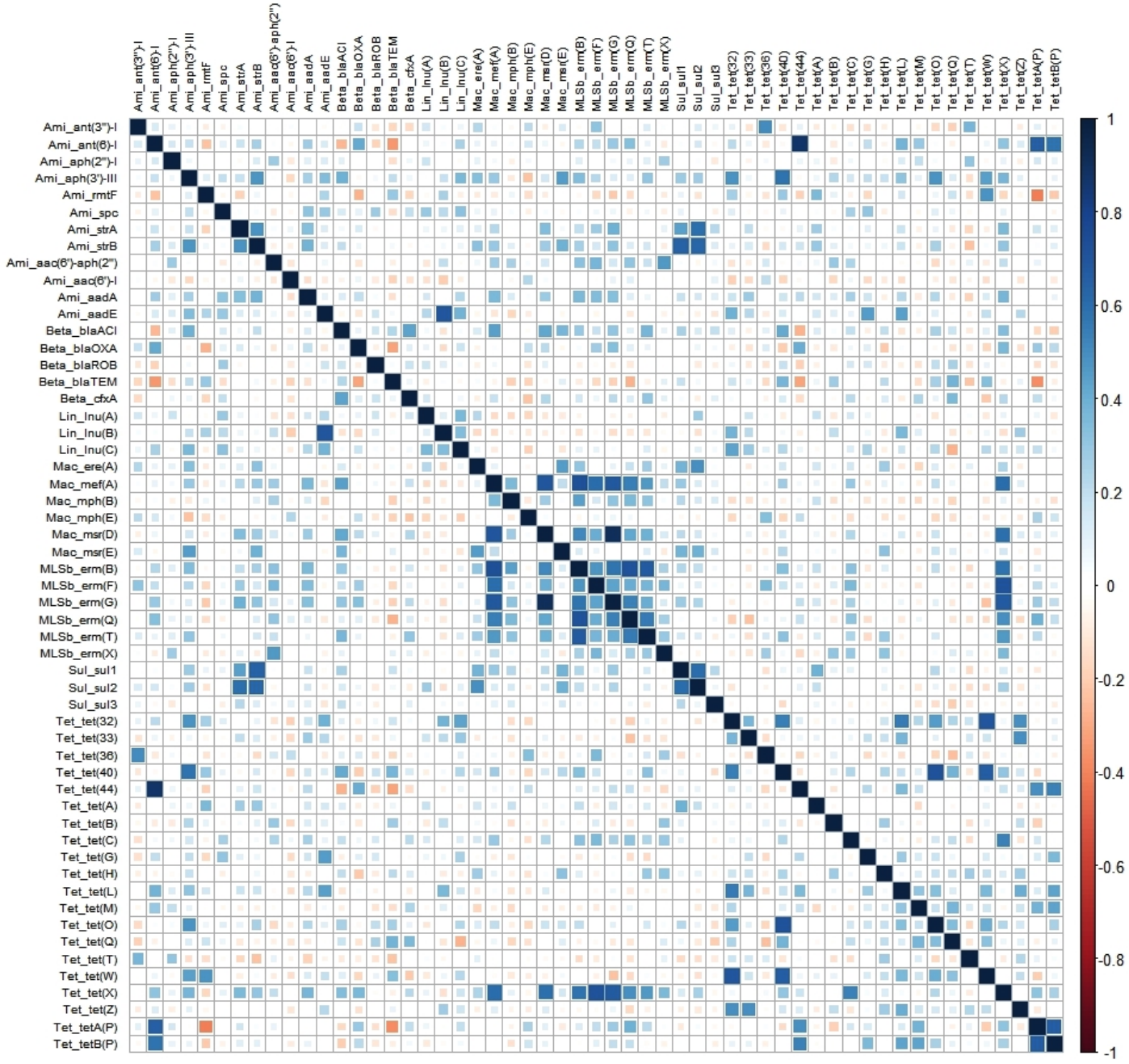


Fig. 5.2.5. Correlation matrix of the resistance genes within the AM-classes; aminoglycosides, beta-lactams, lincosamides, macrolides, MLSb, sulfonamides and tetracyclines, with the significance level set to 0.01.

5.3 Discussion

The main focal point of the third manuscript was the model predicting the effect of altered lifetime AMU in finisher batches on the abundance of AMR genes (AMR abundance) in their gut microbiome close to slaughter at national level. The effect parameters used in the model were based on effect estimates from linear regression models of individual AM-classes determining the quantitative effect of lifetime AMU in finisher batches on AMR abundance in their gut microbiome close to slaughter, i.e. the main goal of this thesis.

The study farms were selected using stratified sampling based on production-type (conventional or organic), the annual number of pigs delivered for slaughter and the annual number of suppliers of pigs. The restrictive selection aimed at obtaining a representative study sample compared with farms producing the majority of pigs slaughtered annually in Denmark, with the predominant supplier paths, which makes it possible to draw inferences from the study sample to the source population. The over representation of relatively large farms was established to study the AMU-AMR associations in farms with a size and production system in alignment with the structural development in pig farms expected by the pig industry in the future.

Several farmers (23%) were uninterested in participating in the study; the reasons for their choice lack of time or financial compensation, have previous had bad experience when participating etc. Therefore, the impact of non-participants compared with our results is unknown. The association between AMU and AMR could potentially be biased due to non-participants. Of those asked to participate, 38% accepted while 23% had altered/ceased their production, and 16% could not be reached.

Structured interviews with detailed farm management questions were carried out in connection with the farm visits. The questions covered four main subjects of interest, **basic information**; production-system and number of pigs per age-group, **production parameter**s; supplier(s) CHR number and number of suppliers per batch, **farm management**; mixing of pigs from separate batches, all-in/all-out managing, cleaning, disinfection, number of days between batches and feed purchase, and **AMU**; farm records and vet instruction to the extent possible. The problem was that these potential risk factors all related to events in the finisher unit, and initial assessments revealed that none of them interacted with the AMU in regression modelling. Consequently, the variables were omitted from all analyses. Hereafter, the effect of lifetime AMU on AMR abundance was only assessed compared with the design variables as confounders, though updated to represent the batch the most. In the assessment of potential risk factors influencing the level of AMR of a finisher batch, a holistic approach rather that the traditional farm level might be a better choice. After omitting all interview data from analyses, the farm-records and vet instruction regarding AMU were used to validate the lifetime AMU.

The finisher batches varied in rearing pathways, however, the pathways of the majority of batches were simple, i.e. pigs in a unit originated from the same farm or from one farm only. The rearing

pathway of two finisher batches stood out, due to the complexity of their pathway that included five and seven farms, respectively (Fig. 5.2.1). Furthermore, in five cases, two finisher batches came from farms owned by one farmer. In addition, it was decided to consider a CHR-farm as an epidemiological unit without considering ownership of supplier farms. Consequently, in four cases, two finisher batches originated from the same supplier farm. Since the farm of origin and farm usage history have been demonstrated to have an effect of AMR abundance (Dawson *et al.*, 1984; Dorado-García *et al.*, 2016), the batches may share similarities.

Lifetime AMU was calculated as the total amount of individual AM-classes throughout the rearing period at dispensing-type level. By doing so, it was not possible to distinguish between differences in AMU within the three rearing periods. That fact that the lifetime AMU does not distinguish between usage within the three rearing periods, known to affect the occurrence of AMR in finishers close to slaughter differently (Callens *et al.*, 2015; Birkegård *et al.*, 2018), may account for some of the non-explained variation in AMR abundance between batches.

The finisher batches were ranked according to their total AMU to display, the differences between the batches usage at age-group level (Fig. 5.2.2.). The first five batches came from farms with organic production that had a small amount of parenteral AMU. Parenteral AMs were used mostly for sows and piglets and to a lesser extent to weaners and finishers, while the peroral AMs were used mainly for weaners and finishers and the smallest amount was used for sows and piglets. Several of the finisher batches were using unexpected large amounts of peroral AMs while in the finisher unit, which were related to farms with finisher systems only. This finding has also been demonstrated in other studies (Hybschmann *et al.*, 2011; van der Fels-Klerx *et al.*, 2011). However, as these farms produced from 3,500 to 28,900 finishers annually, the high usage was not associated with farm size. In contrast, several finisher batches had no usage of peroral AMs with no common features to explain the lack of peroral usage. In general, the usage of peroral AMs was widespread and the peroral macrolide pleuromutilins and tetracyclines were the most used AMs. Although the colistin usage seemed minor, usage occurred in all three rearing periods, which is worrying given the importance of this substance in human medicine (Fig. 5.2.2.).

The estimated effect of AMU on AMR barely changed when the updated design variables were included, indicating that the effect of AMU on AMR at AM-class level is not strongly influenced by farm size and supplier number. However, the design variables are only based on the finisher unit not the entire rearing pathway. The low level of AMR abundance of most AM-classes in the organic production compared with conventional production has been demonstrated (Österberg *et al.*, 2016), and AMR has equally been demonstrated to persist in farms with no AMU (Zhang *et al.*, 2013). The AMR in the organic farms may therefore constitute baseline levels, in that case difficulties could arise if aiming to bring the AMR abundance below the baseline based solely on reducing AMU. The higher level of beta-lactam resistance in the organic farms coincided with parenteral narrow-spectrum penicillin usage as their main drug choice, while the higher level of sulfonamide resistance could not be explained.

The scatterplots of the uni-variable regression models indicated simple linear relationships between lifetime AMU in finisher batches and AMR abundance in their gut microbiome close to slaughter for all AM-classes. Hence, in the multi-variable regression models, the effects of lifetime AMU on AMR abundance were estimated as linear, which was supported by visual inspection of the model's diagnostic plots. As AMU and AMR is low in Denmark, extrapolation of the observed linear effect of lifetime AMU to AMR abundance to higher levels of AMU should be carried out with caution.

Generally, the peroral AMs appeared to affect the AMR abundance far more broadly than the parenteral AMs, which in turn had a higher effect on resistance abundancy. The broader effect of peroral AMs may be due to their widespread but intermittent usage during the weaner and finisher rearing periods. Several studies have assessed the effect of administration routes, and demonstrated that peroral AMs had the most significant effect on AMR (Wiuff *et al.*, 2003; Zhang *et al.*, 2013), though one study found no difference (Græsbøll *et al.*, 2017). The higher effect of parenteral AMs may be the result of environmental contamination of AMs and their metabolites from individually treated pigs, leading to the continuous presence of sub-therapeutic doses (Dawson *et al.*, 1984; Kietzmann *et al.*, 1995; Looft *et al.*, 2012; Singer *et al.*, 2016). Alternatively, the difference between dispensing-type could occur, because they are affecting different resistance genes. In addition, the absorption, distribution and elimination of AM substances may also contribute to the different effect observed between parenteral and peroral AMs on AMR abundance (Wiuff *et al.*, 2003; Zhang *et al.*, 2013). Overall, the results obtained for parenteral AMs were unexpected.

The lacking effect of parenteral extended-spectrum penicillins, macrolides and sulfonamides should be interpreted with caution, as they were used mainly during the piglet-rearing period. The lifetime AMU does not distinguish between usages at different rearing periods. Consequently, usage of these AMs in the finisher unit might have affected resistance abundance. Therefore, this study could not demonstrate that usage of parenteral extended-spectrum penicillins, macrolides and sulfonamides in the piglet-rearing period affects the resistance abundance in their gut microbiome at the time of slaughter.

The wide-ranging co-selection of peroral macrolides in the multi-variable regression models was a finding with parallels to other studies. Rosengren *et al*. (2007) found that the occurrence of sulfamethoxazole and chloramphenicol resistance was six times higher in farms with high macrolide usage compared with farms with no usage of macrolides. Looft *et al*. (2012) also demonstrated the potential for co-selection from a single AM-class usage. Interestingly, it was the most frequently used AMs, peroral macrolides and parenteral and peroral tetracyclines that resulted in the observed widespread co-selection. The increased occurrence of multi-drug AMR and resistance genes affecting several AMs is worrying as it substantially enhances the spread of AMR (Levy and Marshall, 2004; Andersson and Hughes, 2011; Dorado-García *et al.*, 2016).

The underlying effect of parenteral and peroral AMs on individual resistance genes, revealed that peroral macrolide usage affected other AM-classes, though this related mainly to a small number of

resistance genes. Similar findings were shown for the parenteral aminoglycoside usage, which affected a small number of resistance genes in several other AM-classes. The correlation matrix between the abundance of AMR genes, revealed that the deviating behaviour of *tet*(X) was due to the correlation with several macrolide and MLSb genes, and that the lack of effect of the sulfonamide usage on the corresponding genes was probably due to the correlation between *sul1* and *sul2*.

This study included a vast number of correlation and regression analyses at gene level. As a consequence, a high probability of false discovery was expected, which was circumvented by reducing the significance level from 0.05 to 0.01. Despite taking this precaution, the results from these analyses should be viewed as indicative only and as a supporting tool for the assessing and understanding of the quantitative effect of lifetime AMU on AMR abundance.

6. Conclusions and perspectives

This thesis demonstrates that register data can be applied in calculating lifetime AMU in finisher batches close to slaughter independent of rearing site and production system. The developed method revealed that the entire rearing period must be taken into account when studying the effect of lifetime AMU in finishers on AMR abundance in their gut microbiome close to slaughter. Additionally, the thesis provides insight into the limitations and disadvantages of the developed method. Finally, the thesis quantifies the effect of lifetime AMU in finisher batches on AMR abundance in their gut microbiome close to slaughter.

The most time-consuming work in this study concerned quality checking of all the components forming the lifetime AMU as well as the ResFinder's gene allocation to AM-class level.

The first study offers a new method for calculating AMU in finishers based on register data, which estimate the lifetime exposure of finishers from birth piglets to the time of slaughter. The measurement in its current form suffers from the inability to separate the AMU in sows from the AMU in piglets. A separation of the two would have provided the means for biomass adjustments that included both numbers of pigs and their mean weight for each rearing period. By omitting the mean weight of pigs in the lifetime AMU calculations, the usage in finishers may be over-emphasised compared with the number of pigs actually treated. Notwithstanding this issue, the method offers a framework for future development of more sophisticated methods for measuring the lifetime AMU of young production animals across countries.

With advances in inexpensive metagenomics methods, it is expected that these will gradually supersede the traditional cultivation methods. The output of metagenomics methods needs to be translated into meaningful outcomes, e.g. phenotypical resistance and resistance mechanisms, in order to genuinely assess the importance of the outcomes. In this study, the lifetime AMU had a significant effect on the abundance of AMR genes. On the other hand, the resistance genes may not constitute a risk to humans, since the phenotypical resistance obtained by cultivation of *E. coli* compared with any of the approaches of calculating AMU showed no association. A huge task lies ahead, to develop methods that can provide metagenomic data equalling count data from cultivation.

The second study validated the lifetime AMU. In general, the lifetime AMU was improved by altering the method of data transformation, i.e. both accuracy and precision increased, which was additionally confirmed by re-analyses of lifetime AMU and AMR abundance of the ten finisher batches from the previous study. Based on the results, the study demonstrated that by means of data transformation the CHR, PMD and VetStat databases can be used to calculate lifetime AMU that mimics the "true" usage accurately and with modest precision. In addition, the reliability coefficients revealed that the calculations of the daily amounts of AMs used per pig underestimate the usage independent of the smoothing method.

In Denmark, VetStat, PMD and CHR give access to data at farm unit level, thereby providing unique opportunities to study AMU across farms and units, and its effect on AMR. In addition, the data is easily obtained for the entire population of pigs. However, several adverse issues were encountered that potentially explain the modest precision of the most valid transformation method. Minor efforts could be usefully directed towards ensuring correct data entries, which would immensely improve the lifetime AMU.

These results highlight the general importance of valid data in epidemiological studies in order to obtain unbiased quantitative estimates of effects and reduce the risk of erroneous conclusions, i.e. access to accurate and precise data when calculating the lifetime AMU is key to obtain reliable regression coefficients of the effect of AMU on AMR. Even though the validation study sample was small, it provides sufficient knowledge to improve the lifetime AMU. Equal importantly, the study gave insight into vet instructions and usage patterns in farms to be considered when associations between AMU and AMR are assessed. Nonetheless, it would be relevant to perform a comprehensive study to gain knowledge of the prevalence of the encountered issues, as this will provide trustworthy measurement errors of the effect estimates, thus, their reliability.

The third study assessed the effect of lifetime AMU on AMR abundance. Overall, the study generated knowledge of the quantitative effect of parenteral and peroral lifetime AMU on AMR abundance, at AM-class level, in the gut microbiome of finisher batches close to slaughter. Although significant effects of lifetime AMU were found for AMR abundance of every AM-class resistance investigated, the AMUs could explain only between 9% - 52% of the variation in AMR. The inability to assess risk factors was a huge step backwards. Therefore, a holistic approach should be employed when evaluating risk factors related to finisher batches, e.g., inherited AMR level as a result of previous AMU at rearing sites and the effect of mixing pigs with different AMR levels.

Even though models do not confirm causal relationships, rather, it assumes causal links and then test how strong they would be if the model were a correct representation of reality (Martin, 2014), our results combined with knowledge from similar studies suggest causal associations. Subsequently, our results provide additional knowledge in the understanding of the complexity of AMR emergence and spread across the pig population.

The effect estimates from the quantification study combined with detailed data on lifetime AMU of the majority of finishers in Denmark have been applied as inputs to the development of a national scale predictive model, which enables testing of different AMU scenarios prior to potentially selecting the most efficient intervention. The predictive model will also facilitate predictions of the amount of AMU reduction required to reach a desired reduction in AMR. In itself, this provides a significant tool for the Danish authorities and other stakeholders, but it also provides an example of what is feasible and what data will be needed in order to provide guidance for major political and targeted interventions regarding production animals globally. Thus, the thesis has provided a framework for further development that might eventually assist in reducing AMR and safeguarding AMs for the future.

Acknowledgements

First, I wish to express my sincere thanks to Håkan for his constructive guidance, helpful teaching, inspirational motivation and convincing discussions at all times, let alone the sincere and valuable support and encouragement extended to me throughout this academic journey. I could not have imagined having a better advisor and coach for my PhD study. I am extremely thankful to Leonardo for his unlimited help, brainstorming and lengthy discussions always accompanied by Star Wars references. Thank you for the joy and laughter. I also thank Yvonne for her guidance and help during the study. I am grateful to my supervisors for their guidance and critical review of my work, and the co-authors for their valuable input and contributions.

I am thankful to my many former colleagues at the Danish Zoonosis Centre and the Epidemiology and Risk Modelling group of the National Food Institute for their introduction to the field of academic research wrapped in encouragement and embracing support. I am thankful to all my colleagues at the Research Group for Genomic Epidemiology for their keen interest, sharing of outstanding expertise and technical support. I am grateful to my fellow PhD students for always including me despite the age difference.

Thanks to colleagues and stakeholders of "Vetforlig II" for the stimulating discussions and productive collaboration. Many thanks to the VetStat study group (Amanda Brinch Kruse, Camilla Birkegård, Gitte Blach Nielsen, Leonardo de Knegt, Mette Fertner and Nanna Dupont) for their sharing of knowledge and views on the challenges encountered working with VetStat. A special thanks to my friend Hanne Rosenquist, for the motivation and the constructive inputs to the thesis.

I am forever in debt to my loving mother Birta Jakobsen, for always being there - believing in me. Profound thanks for showing me the sky without limits, for inspiring me to try, and for making me trust in hard work to fulfil my dreams. To my partner in crime, Søren, and our amazing offspring, Tobias and Mads, heartfelt thanks for loving and supporting me through this journey and my life in general. Last but not least, many thanks to my family and friends for being supportive all the way.

References

Aarestrup, F. M. and Wegener, H. C. (1999) 'The effects of antibiotic usage in food animals on the development of antimicrobial resistance of importance for humans in *Campylobacter* and *Escherichia coli*', *Microbes and Infection*, 1(8), pp. 639–644.

Aarestrup, F. M. *et al.* (2001) 'Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark', *Antimicrobial Agents and Chemotherapy*, 45(7), pp. 2054–2059. doi: 10.1128/AAC.45.7.2054-2059.2001 [doi].

Aarestrup, F. M. *et al.* (2006) 'First description of blaCTX-M-1-carrying *Escherichia coli* isolates in Danish primary food production', *Journal of Antimicrobial Chemotherapy*, 57(6), pp. 1258–1259.

Aarestrup, F. M. *et al.* (2010) 'Changes in the use of antimicrobials and the effects on productivity of swine farms in Denmark', *American Journal of Veterinary Research*, 71(7), pp. 726–733.

Aarestrup, F. M. (2015) 'The livestock reservoir for antimicrobial resistance: a personal view on changing patterns of risks, effects of interventions and the way forward.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 370(1670). doi: 10.1098/rstb.2014.0085.

Abatih, E. N. *et al.* (2009) 'Impact of antimicrobial usage on the transmission dynamics of antimicrobial resistant bacteria among pigs', *Journal of Theoretical Biology*, 256(4), pp. 561–573.

Agersø¸, Y. and Aarestrup, F. M. (2013) 'Voluntary ban on cephalosporin use in Danish pig production has effectively reduced extended-spectrum cephalosporinase-producing *Escherichia coli* in slaughter pigs', *Journal of Antimicrobial Chemotherapy*, 68(3), pp. 20569–20572.

Akwar, H. T. *et al.* (2008) 'Associations of antimicrobial uses with antimicrobial resistance of fecal *Escherichia coli* from pigs on 47 farrow-to-finish farms in Ontario and British Columbia', *Canadian Journal of Veterinary Research*, 72(2), pp. 202–210.

Alban, L., Petersen, J. and Busch, M. (2015) 'A comparison between lesions found during meat inspection of finishing pigs raised under organic/free-range conditions and conventional, indoor conditions', *Porcine Health Management*. BioMed Central, 1(1), p. 4. doi: 10.1186/2055-5660-1-4.

Andersen, V. D. *et al.* (2015) 'The use of third and fourth generation cephalosporins affects the occurrence of extended-spectrum cephalosporinase-producing *Escherichia coli* in Danish pig herds', *Veterinary Journal*, 204(3), pp. 345–350. doi: 10.1016/j.tvjl.2015.03.014.

Andersen, V. D. *et al.* (2017) 'The association between measurements of antimicrobial use and resistance in the faeces microbiota of finisher batches', *Epidemiology and Infection*, 145(13), pp. 2827–2837. doi: 10.1017/S0950268817001285.

Andersson, D. I. and Hughes, D. (2010) 'Antibiotic resistance and its cost: Is it possible to reverse resistance?', *Nature Reviews Microbiology*, 8(4), pp. 260–271.

Andersson, D. I. and Hughes, D. (2011) 'Persistence of antibiotic resistance in bacterial populations', *FEMS microbiology reviews*, 35(5), pp. 901–911.

Andraud, M. *et al.* (2011) 'Estimation of transmission parameters of a fluoroquinolone-resistant *Escherichia coli* strain between pigs in experimental conditions', *Veterinary Research*, 42(1).

Armstrong, B. G. (1998) 'Effect of measurement error on epidemiological studies of environmental and occupational exposures', *Occupational and Environmental Medicine*, 55(10), pp. 651–656. doi: 10.1136/oem.55.10.651.

Backhans, A. *et al.* (2016) 'Antimicrobial use in Swedish farrow-to-finish pig herds is related to farmer characteristics', *Porcine Health Management*, 2(1), p. 18. doi: 10.1186/s40813-016-0035-0.

Bager, F. *et al.* (1997) 'Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms.', *Preventive Veterinary Medicine*, 31(1–2), pp. 95–112.

Bager, F. (2000) 'DANMAP: Monitoring antimicrobial resistance in Denmark', *International Journal of Antimicrobial Agents*, 14(4), pp. 271–274. doi: 10.1016/S0924-8579(00)00135-7.

Barlow, M. (2009) 'What Antimicrobial Resistance Has Taught Us About Horizontal Gene Transfer', in Gogarten, M. B., Gogarten, J. P., and Olendzenski, L. C. (eds) *Methods in molecular biologi*, pp. 397–411. doi: DOI 10.1007/978-1-60327-853-9

Berendsen, B. J. A. *et al.* (2015) 'The analysis of animal faeces as a tool to monitor antibiotic usage.', *Talanta*, 132, pp. 258–268. doi: 10.1016/j.talanta.2014.09.022.

Bergenholtz, R. D. *et al.* (2009) 'Characterization of genetic determinants of extended-spectrum cephalosporinases (ESCs) in *Escherichia coli* isolates from Danish and imported poultry meat', *Journal of Antimicrobial Chemotherapy*, 64(1), pp. 207–209.

Bigras-Poulin, M. *et al.* (2007) 'Relationship of trade patterns of the Danish swine industry animal movements network to potential disease spread', *Preventive Veterinary Medicine*, 80(2–3), pp. 143–165. doi:

10.1016/j.prevetmed.2007.02.004.
Birkegård, A. C., *et al.* (2017) 'Association between selected antimicrobial resistance genes and antimicrobial exposure in Danish pig farms', *Scientific Reports*, 7(1). doi: 10.1038/s41598-017-10092-9.
Birkegård, A. C., *et al.* (2017a) 'Computional algorithm for lifetime exposure to antimicrobials in pigs using register data—The LEA algorithm', *Preventive Veterinary Medicine*, 146, pp. 173–180. doi: 10.1016/j.prevetmed.2017.08.008.
Birkegård, A. C. *et al.* (2018) 'Persistence of antimicrobial resistance genes from sows to finisher pigs', *Preventive Veterinary Medicine*, 149, pp. 10–14. doi: 10.1016/j.prevetmed.2017.11.007.
Bland, J. M. and Altman, D. G. (2010) 'Statistical methods for assessing agreement between two methods of clinical measurement.', *The Lancet*, 47(8), pp. 307–310. doi: 10.1016/j.ijnurstu.2009.10.001.
van Boeckel, T. P. *et al.* (2015) 'Global trends in antimicrobial use in food animals', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1503141112.
Bondt, N. *et al.* (2013) 'Comparing antimicrobial exposure based on sales data', *Preventive Veterinary Medicine*, 108(1), pp. 10–20. doi: 10.1016/j.prevetmed.2012.07.009.
Burow, E. *et al.* (2014) 'Oral antimicrobials increase antimicrobial resistance in porcine *E. coli* - a systematic review.', *Preventive Veterinary Medicine*, 113(4), pp. 364–375. doi: 10.1016/j.prevetmed.2013.12.007.
Callens, B. *et al.* (2012) 'Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds', *Preventive Veterinary Medicine*, 106(1), pp. 53–62.
Callens, B. *et al.* (2015) 'Presence of Antimicrobial Resistance and Antimicrobial Use in Sows Are Risk Factors for Antimicrobial Resistance in Their Offspring', *Microbial Drug Resistance*, 21(1), pp. 50–58. doi: 10.1089/mdr.2014.0037.
Callens, B. *et al.* (2017) 'Associations between a decreased veterinary antimicrobial use and resistance in commensal Escherichia coli from Belgian livestock species (2011–2015)', *Preventive Veterinary Medicine*. doi: 10.1016/j.prevetmed.2017.10.013.
Campbell, J. M., Crenshaw, J. D. and Polo, J. (2013) 'The biological stress of early weaned piglets.', *Journal of Animal Science and Biotechnology*, 4(1), p. 19. doi: 10.1186/2049-1891-4-19.
Cantón, R. *et al.* (2011) 'Co-resistance: an opportunity for the bacteria and resistance genes', *Current Opinion in Pharmacology*, 11(5), pp. 477–485.
Cavaco, L. M. *et al.* (2008) 'Selection and persistence of CTX-M-producing *Escherichia coli* in the intestinal flora of pigs treated with amoxicillin, ceftiofur, or cefquinome', *Antimicrobial Agents and Chemotherapy*, 52(10), pp. 3612–3616.
Cavaco, L. M. *et al.* (2010) 'Cloning and occurrence of czrC, a gene conferring cadmium and zinc resistance in methicillin-resistant Staphylococcus aureus CC398 isolates', *Antimicrobial Agents and Chemotherapy*, 54(9), pp. 3605–3608. doi: 10.1128/AAC.00058-10.
Cavaco, L. M., Hasman, H. and Aarestrup, F. M. (2011) 'Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance', *Veterinary Microbiology*, 150(3–4), pp. 344–348.
CDC (2013) 'Antibiotic resistance threats in the United States, 2013', doi: CS239559-B (Accessed: 15 January 2018).
Chauvin, C. *et al.* (2001) 'The crucial question of standardisation when measuring drug consumption', *Veterinary Research*, pp. 533–543. doi: 10.1051/vetres:2001145.
Christiansen, M. G. (2014) 'No 1429: The Structural Development in the Danish Pig Production 2014 [in Danish]', *SEGES*.
Available at: http://svineproduktion.dk/Publikationer/Kilder/Notater/2014/1429.aspx (Accessed: 15 January 2018).
Collineau, L. *et al.* (2017) 'Guidance on the Selection of Appropriate Indicators for Quantification of Antimicrobial Usage in Humans and Animals', *Zoonoses and Public Health*, 64(3), pp. 165–184. doi: 10.1111/zph.12298.
Damgaard, B. M., Studnitz, M. and Jensen, K. H. (2009) 'The effect of continuous grouping of pigs in large groups on stress response and haematological parameters', *Livestock Science*, 121(1), pp. 137–140. doi: 10.1016/j.livsci.2008.05.011.
Danish Crown (2017) Danish Crown Kontrakts [in Danish only]. Available at: https://www.danishcrown.dk/ejer/svineleverandoer/danish-crown-afregning/danish-crown-kontrakt/ (Accessed: 15 January 2018).
DANMAP (2010) Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark, 2009.
Available at: http://www.danmap.org/Downloads/Reports.aspx / (Accessed: 15 January 2018).
DANMAP (2012) Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark, 2011.
Available at: http://www.danmap.org/Downloads/Reports.aspx / (Accessed: 15 January 2018).
DANMAP (2017) Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark, 2016.

Available at: http://www.danmap.org/Downloads/Reports.aspx / (Accessed: 15 January 2018).
Davies, J. and Davies, D. (2010) 'Origins and evolution of antibiotic resistance', *Microbiology and Molecular Biology Reviews*, 74(3), pp. 417–433.
Dawson, K. A. *et al.* (1984) 'Antibiotic resistance in anaerobic and coliform bacteria from the intestinal tract of swine fed therapeutic and subtherapeutic concentrations of chlortetracycline.', *Journal of Animal Science*, 58(1), pp. 123–131.
Dewulf, J. *et al.* (2007) 'Tetracycline-resistance in lactose-positive enteric coliforms originating from Belgian fattening pigs: Degree of resistance, multiple resistance and risk factors', *Preventive Veterinary Medicine*, 78(3–4), pp. 339–351. doi: 10.1016/j.prevetmed.2006.11.001.
Dorado-García, A. *et al.* (2016) 'Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the Netherlands', *Journal of Antimicrobial Chemotherapy*, 71(12), pp. 3607–3619. doi: 10.1093/jac/dkw308.
Dunlop, R. H. *et al.* (1998) 'Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada.', *Preventive Veterinary Medicine*, 34, pp. 283–305. doi: 10.1016/S0167-5877(97)00095-0.
Dupont, N. *et al.* (2016) 'Reporting the national antimicrobial consumption in Danish pigs: Influence of assigned daily dosage values and population measurement', *Acta Veterinaria Scandinavica*, 58(1). doi: 10.1186/s13028-016-0208-5.
Dupont, N. *et al.* (2017) 'Improving institutional memory on challenges and methods for estimation of pig herd antimicrobial exposure based on data from the Danish Veterinary Medicines Statistics Program (VetStat)'. Available at: http://arxiv.org/abs/1705.08663 (Accessed: 15 January 2018).
DVFA (2005) Evidence-based Prudent Use Guidelines for Antimicrobial Treatment of Pigs. Available at: https://www.foedevarestyrelsen.dk/english/Animal/AnimalHealth/Veterinary_medicine/Pages/Evidence_based_prudent_use_guidelines_for_antimicrobial_treatment_of_pigs.aspx (Accessed: 15 January 2018).
DVFA (2016) 'Special provisions for the reduction of the consumption of antibiotics in pig holdings (the yellow card initiative)', p. 4.
Available at: https://www.foedevarestyrelsen.dk/english/SiteCollectionDocuments/Dyrevelfaerd og veterinaermedicin/Veterinærmedicin/Yellow Card, English version, 180517.pdf (Accessed: 15 January 2018).
DVFA (2017) Guidance for case processing regarding exceedance of limit values for antibiotic consumption [in Danish only]. Available at: https://www.foedevarestyrelsen.dk/SiteCollectionDocuments/Dyrevelfaerd%20og%20veterinaermedicin/Veterin%C3%A6rmedicin/Antibiotika/310317%20Gult%20kort%20Vejledning%20ver%202.7.pdf (Accessed: 15 January 2018).
ECDC/EMEA (2009) Joint Technical Report: The bacterial challenge: time to react. Available at: http://ecdc.europa.eu/en/publications/_layouts/forms/Publication_DispForm.aspx?ID=199&List=4f55ad51-4aed-4d32-b960-af70113dbb90 (Accessed: 11 January 2017).
EFSA (2008) 'Harmonised monitoring of antimicrobial resistance in *Salmonella* and *Campylobacter* isolates from food animals in the European Union.', *Clinical Microbiology and Infection*, 14(1469–691), pp. 522–533.
EFSA/ECDC (2016) The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014, *EFSA Journal*. doi: 10.2903/j.efsa.2016.4380.
Ekkel, E. D. *et al.* (1996) 'Health, Welfare, and Productivity of Pigs Housed under Specifi c-Stress-Free Conditions in Comparison with Two-Site Systems', *Journal of Animal Science*, 74(9), pp. 2081–2087.
Emanuelson, U. and Egenvall, A. (2014) 'The data - sources and validation.', *Preventive Veterinary Medicine*, 113(3), pp. 298–303. doi: 10.1016/j.prevetmed.2013.10.002.
Emborg, H. *et al.* (2001) 'The effect of discontinuing the use of antimicrobial growth promoters on the productivity in the Danish broiler production.', *Preventive Veterinary Medicine*, 50(1–2), pp. 53–70.
Emborg, H. D. *et al.* (2007) 'Tetracycline consumption and occurrence of tetracycline resistance in Salmonella typhimurium phage types from Danish pigs', *Microbial Drug Resistance*, 13(4), pp. 289–294. doi: 10.1089/mdr.2007.746.
European Council (1970) 'Council Directive of 23 November 1970 concerning additives in feeding-stuffs (70/524/EEC)', *Official Journal of the European Communities*, pp. 1–17. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31970L0524&from=en (Accessed: 15 January 2018).
van der Fels-Klerx, H. J. *et al.* (2011) 'Farm factors associated with the use of antibiotics in pig production', *Journal of Animal Science*, 89(6), pp. 1922–1929. doi: 10.2527/jas.2010-3046.
Fey, P. D. *et al.* (2000) 'Ceftriaxone-resistant Salmonella infection acquired by a child from cattle', *New England Journal of Medicine*, 342(17), pp. 1242–1249.
Frimodt-Møller, N. (2004) 'Microbial threat - The Copenhagen recommendations initiative of the EU', *Journal of Veterinary Medicine Series B: Infectious Diseases and Veterinary Public Health*, 51(8–9), pp. 400–402.

Gibbons, J. F. *et al.* (2016) 'Antimicrobial Resistance of Faecal *Escherichia coli* Isolates from Pig Farms with Different Durations of In-feed Antimicrobial Use', *Zoonoses and Public Health*, 63(3). doi: 10.1111/zph.12225.
Græsbøll, K. *et al.* (2017) 'Effect of tetracycline dose and treatment mode on selection of resistant coliform bacteria in nursery pigs', *Applied and Environmental Microbiology*, 83(12). doi: 10.1128/AEM.00538-17.
Hammerum, A. M. *et al.* (2007) 'Danish integrated antimicrobial resistance monitoring and research program.', *Emerging Infectious Diseases*, 13(11), pp. 1632–9. doi: 10.3201/eid1311.070421.
Hammerum, A. M., Lester, C. H. and Heuer, O. E. (2010) 'Antimicrobial-resistant *enterococci* in animals and meat: A human health hazard?', *Foodborne Pathogens and Disease*, 7(10), pp. 1137–1146. doi: 10.1089/fpd.2010.0552.
Hammerum, A. M. *et al.* (2014) 'Characterization of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins', *Journal of Antimicrobial Chemotherapy*, 69(10). doi: 10.1093/jac/dku180.
Hansen, K. H. *et al.* (2013) 'Carriage and fecal counts of CTX-M-Producing *Escherichia coli* in Pigs: A longitudinal study', *Applied and Environmental Microbiology*, 79(3), pp. 20794–20798.
Hasman, H. and Aarestrup, F. M. (2005) 'Relationship between copper, glycopeptide, and macrolide resistance among *Enterococcus faecium* strains isolated from pigs in Denmark between 1997 and 2003', *Antimicrobial Agents and Chemotherapy*, 49(1), pp. 454–456. doi: 10.1128/AAC.49.1.454-456.2005.
Holman, D. B. and Chénier, M. R. (2013) 'Impact of subtherapeutic administration of tylosin and chlortetracycline on antimicrobial resistance in farrow-to-finish swine', *FEMS Microbiology Ecology*, 85(1), pp. 1–13. doi: 10.1111/1574-6941.12093.
Holman, D. B. and Chénier, M. R. (2015) 'Antimicrobial use in swine production and its effect on the swine gut microbiota and antimicrobial resistance', *Canadian Journal of Microbiology*, 61(11), pp. 785–798. doi: 10.1139/cjm-2015-0239.
Holmes, A. H. *et al.* (2016) 'Understanding the mechanisms and drivers of antimicrobial resistance', *The Lancet*, 387(10014), pp. 176–187. doi: 10.1016/S0140-6736(15)00473-0.
Houe, H., Gardner, I. A. and Nielsen, L. R. (2011) 'Use of information on disease diagnoses from databases for animal health economic, welfare and food safety purposes: strengths and limitations of recordings', *Acta Veterinaria Scandinavica*, 53(suppl 1), p. S7.
Hybschmann, G. K. *et al.* (2011) 'Herd-level risk factors for antimicrobial demanding gastrointestinal diseases in Danish herds with finisher pigs A register-based study', *Preventive Veterinary Medicine*, 98(2–3), pp. 190–197. doi: 10.1016/j.prevetmed.2010.10.005.
Jensen, H. H. and Hayes, D. J. (2014) 'Impact of Denmark's ban on antimicrobials for growth promotion', *Current Opinion in Microbiology*, pp. 30–36. doi: 10.1016/j.mib.2014.05.020.
Jensen, V. F. *et al.* (2006) 'Correlation between apramycin and gentamicin use in pigs and an increasing reservoir of gentamicin-resistant Escherichia coli', *Journal of Antimicrobial Chemotherapy*, 58(1), pp. 101–107.
Jensen, V. F., Jacobsen, E. and Bager, F. (2004) 'Veterinary antimicrobial-usage statistics based on standardized measures of dosage', *Preventive Veterinary Medicine*, 64(2–4), pp. 201–215. doi: 10.1016/j.prevetmed.2004.04.001.
Jensen, V. F. *et al.* (2014) 'Temporal relationship between decrease in antimicrobial prescription for Danish pigs and the "Yellow Card" legal intervention directed at reduction of antimicrobial use', *Preventive Veterinary Medicine*, 117(3–4). doi: 10.1016/j.prevetmed.2014.08.006.
Jessen, O. (2015) 'No 1523: National pig productivity avarage 2014 [in Danish only]'. SEGES, pp. 1–16. Available at: http://vsp.lf.dk/~/media/Files/PDF - Publikationer/Notater 2015/Notat_1523.pdf (Accessed: 15 January 2018).
Jiang, X. *et al.* (2017) 'Dissemination of antibiotic resistance genes from antibiotic producers to pathogens', *Nature Communications*, 8. doi: 10.1038/ncomms15784.
Jørgensen, C. J. *et al.* (2007) 'Occurrence of CTX-M-1-producing *Escherichia coli* in pigs treated with ceftiofur [3]', *Journal of Antimicrobial Chemotherapy*, 59(5), pp. 1040–1042. doi: 10.1093/jac/dkm075.
Kietzmann, M. *et al.* (1995) 'Drug residues in untreated swine.', *DTW. Deutsche Tierarztliche Wochenschrift*, 102(11), pp. 441–2.
Kobayashi, T. *et al.* (2007) 'Molecular evidence for the ancient origin of the ribosomal protection protein that mediates tetracycline resistance in bacteria', *Journal of Molecular Evolution*, 65(3), pp. 228–235. doi: 10.1007/s00239-007-9006-z.
Kruse, A. B. *et al.* (2017) 'No Clear Effect of Initiating Vaccination against Common Endemic Infections on the Amounts of Prescribed Antimicrobials for Danish Weaner and Finishing Pigs during 2007–2013', *Frontiers in Veterinary Science*. doi: 10.3389/fvets.2016.00120.
Laxminarayan, R. *et al.* (2013) 'Antibiotic resistance—the need for global solutions', *The Lancet Infectious Diseases*, 13(12), pp. 1057–1098.
Laxminarayan, R. *et al.* (2016) 'Access to effective antimicrobials: a worldwide challenge.', *The Lancet (London, England)*, 387(10014), pp. 168–175. doi: 10.1016/S0140-6736(15)00474-2.
Levy, S. B. and Marshall, B. (2004) 'Antibacterial resistance worldwide: Causes, challenges and responses', *Nature*

medicine, 10(12 Suppl.), pp. 122–129.
Literak, I. *et al.* (2009) 'Highly variable patterns of antimicrobial resistance in commensal *Escherichia coli isolates* from pigs, sympatric rodents, and flies', *Microbial Drug Resistance*, 15(3), pp. 229–237. doi: 10.1089/mdr.2009.0913.
Looft, T. *et al.* (2012) 'In-feed antibiotic effects on the swine intestinal microbiome', *Proceedings of the National Academy of Sciences of the United States of America*, 109(5), pp. 1691–1696. doi: 10.1073/pnas.1120238109.
Luby, E. *et al.* (2016) 'Molecular Methods for Assessment of Antibiotic Resistance in Agricultural Ecosystems: Prospects and Challenges', *Journal of Environment Quality*, 45(2), p. 441. doi: 10.2134/jeq2015.07.0367.
Laanen, M. *et al.* (2013) 'Relationship between biosecurity and production/antimicrobial treatment characteristics in pig herds', *Veterinary Journal*, 198(2), pp. 508–512. doi: 10.1016/j.tvjl.2013.08.029.
Marshall, B. M. and Levy, S. B. (2011) 'Food animals and antimicrobials: Impacts on human health.', *Clinical Microbiology Reviews. American Society for Microbiology*, 24(4), pp. 718–733. doi: 10.1128/CMR.00002-11.
Martel, J.-L. *et al.* (2001) 'New trends in regulatory rules and surveillance of antimicrobial resistance in bacteria of animal origin', *Veterinary Research*, 32(3–4), pp. 381–392. doi: 10.1051/vetres:2001131.
Martin, W. (2014) 'Making valid causal inferences from observational data', *Preventive Veterinary Medicine*, 113(3), pp. 281–297. doi: 10.1016/j.prevetmed.2013.09.006.
Martinez, J. L., Coque, T. M. and Baquero, F. (2015) 'What is a resistance gene? Ranking risk in resistomes', *Nature Reviews Microbiology*, 13(2), pp. 116–123. doi: 10.1038/nrmicro3399.
Mathew, A. G. *et al.* (2003) 'Characterization of resistance patterns and detection of apramycin resistance genes in Escherichia coli isolated from swine exposed to various environmental conditions', *International Journal of Food Microbiology*, 89(1), pp. 11–20. doi: 10.1016/S0168-1605(03)00124-7.
Mathew, A. G. *et al.* (2005) 'Effects of Antibiotic Use in Sows on Resistance of *E. coli* and *Salmonella enterica* Typhimurium in Their Offspring', *Foodborne Pathogens and Disease*, 2(3), pp. 212–220. doi: 10.1089/fpd.2005.2.212.
Merlot, E., Meunier-Salaün, M.-C. and Prunier, A. (2004) 'Behavioural, endocrine and immune consequences of mixing in weaned piglets', *Applied Animal Behaviour Science*, 85(3–4), pp. 247–257. doi: 10.1016/j.applanim.2003.11.002.
Moodley, A. and Guardabassi, L. (2009) 'Transmission of IncN Plasmids Carrying bla(CTX-M-1) between Commensal *Escherichia coli* in Pigs and Farm Workers', *Antimicrobial Agents and Chemotherapy*, 53(4), pp. 1709–1711.
Munk, P. *et al.* (2017) 'A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds', *Journal of Antimicrobial Chemotherapy*, 72(2). doi: 10.1093/jac/dkw415.
Nielsen, G. B. (2015) Oral communication: Antimicrobial usage in the pig production.
O'Neill, J. (2016) 'Tackling drug-resistant infections globally: final report and recommendations', *Review on Antimicrobial Resistance*, (May), p. 84. doi: 10.1016/j.jpha.2015.11.005.
Postma, M. *et al.* (2015) 'Assigning defined daily doses animal: A European multi-country experience for antimicrobial products authorized for usage in pigs', *Journal of Antimicrobial Chemotherapy*, 70(1), pp. 294–302. doi: 10.1093/jac/dku347.
Postma, M. *et al.* (2016) 'Evaluation of the relationship between the biosecurity status, production parameters, herd characteristics and antimicrobial usage in farrow-to-finish pig production in four EU countries', *Porcine Health Management*, 2(1), p. 9. doi: 10.1186/s40813-016-0028-z.
Postma, M. *et al.* (2016a) 'The biosecurity status and its associations with production and management characteristics in farrow-to-finish pig herds', *Animal*, 10(3), pp. 478–489. doi: 10.1017/S1751731115002487.
Rhouma, M., Beaudry, F. and Letellier, A. (2016) 'Resistance to colistin: what is the fate for this antibiotic in pig production?', *International Journal of Antimicrobial Agents*. doi: 10.1016/j.ijantimicag.2016.04.008.
Rojo-Gimeno, C. *et al.* (2016) 'Farm-economic analysis of reducing antimicrobial use whilst adopting improved management strategies on farrow-to-finish pig farms', *Preventive Veterinary Medicine*, 129. doi: 10.1016/j.prevetmed.2016.05.001.
Rosengren, L. B. *et al.* (2007) 'Associations between feed and water antimicrobial use in farrow-to-finish swine herds and antimicrobial resistance of fecal Escherichia coli from grow-finish pigs', *Microbial Drug Resistance*, 13(4), pp. 261–270.
Schmidt, G. V. *et al.* (2015) 'Sampling and pooling methods for capturing herd level antibiotic resistance in swine feces using qPCR and CFU approaches', *PLoS ONE*, 10(6). doi: 10.1371/journal.pone.0131672.
Schuppers, M. E. *et al.* (2005) 'Clinical herd health, farm management and antimicrobial resistance in Campylobacter coli on finishing pig farms in Switzerland.', *Preventive Veterinary Medicine*, 69(3–4), pp. 189–202. doi: 10.1016/j.prevetmed.2005.02.004.
SEGES (2000) ANNUAL REPORT 1999. Available at: http://svineproduktion.dk/Om-os/Aarsberetninger (Accessed: 15 January 2018).
SEGES (2011) ANNUAL REPORT 2010. Available at: http://svineproduktion.dk/Om-os/Aarsberetninger (Accessed: 15 January 2018).
SEGES (2013) 'Guidelines on good antibiotic practice'. Available at:

http://svineproduktion.dk/viden/i-stalden/management/manualer/antibiotika (Accessed: 15 January 2018).
SEGES (2016) ANNUAL REPORT 2015. Available at: http://svineproduktion.dk/Om-os/Aarsberetninger (Accessed: 15 January 2018).
DAFC (2017) Statistics 2016 pigmeat. Available at: http://agricultureandfood.dk/prices-statistics/annual-statistics (Accessed: 15 January 2018).
Silver, L. L. (2011) 'Challenges of antibacterial discovery.', *Clinical Microbiology Reviews. American Society for Microbiology*, 24(1), pp. 71–109. doi: 10.1128/CMR.00030-10.
Singer, A. C. *et al.* (2016) 'Review of antimicrobial resistance in the environment and its relevance to environmental regulators', *Frontiers in Microbiology*, 7: 1728. doi: 10.3389/fmicb.2016.01728.
Sjölund, M. *et al.* (2015) 'Antimicrobial usage in 60 Swedish farrow-to-finish pig herds', *Preventive Veterinary Medicine*, 121(3), pp. 257–264.
Song, J. *et al.* (2017) 'Comparison of Metals and Tetracycline as Selective Agents for Development of Tetracycline Resistant Bacterial Communities in Agricultural Soil', *Environmental Science & Technology*, 51(5), pp. 3040–3047. doi: 10.1021/acs.est.6b05342.
Speksnijder, D. C. *et al.* (2015) 'Reduction of veterinary antimicrobial use in the Netherlands. The dutch success model', *Zoonoses and Public Health*, 62(s1), pp. 79–87. doi: 10.1111/zph.12167.
Stege, H. *et al.* (2003) 'VETSTAT-the Danish system for surveillance of the veterinary use of drugs for production animals', *Preventive Veterinary Medicine*, 57(3), pp. 105–115.
Swann, M. M. *et al.* (1969) Joint Committee on the use of antibiotics in animal husbandry and veterinary medicine (Swann Report). London, UK, pp 1-83.
Sørensen, H. T., Sabroe, S. and Olsen, J. (1996) 'A framework for evaluation of secondary data sources for epidemiological research', *International Journal of Epidemiology*, 25(2), pp. 435–442.
Taverne, F. *et al.* (2015) 'Influence of applying different units of measurement on reporting antimicrobial consumption data for pig farms', *BMC Veterinary Research*, 11: 250. doi: 10.1186/s12917-015-0566-7.
Taylor, N. M. *et al.* (2009) 'Farm-level risk factors for fluoroquinolone resistance in E. coli and thermophilic *Campylobacter* spp. on finisher pig farms.', *Epidemiology and Infection*, 137(8), pp. 1121–1134. doi: 10.1017/S0950268808001854.
Timmerman, T. *et al.* (2006) 'Quantification and evaluation of antimicrobial drug use in group treatments for fattening pigs in Belgium', *Preventive Veterinary Medicine*, 74(4), pp. 251–263. doi: 10.1016/j.prevetmed.2005.10.003.
Trauffler, M. *et al.* (2014) 'Antimicrobial drug use in Austrian pig farms: plausibility check of electronic on-farm records and estimation of consumption', *Veterinary Record*, p. vetrec-2014-102520. doi: 10.1136/vr.102520.
Turnidge, J. and Paterson, D. L. (2007) 'Setting and revising antibacterial susceptibility breakpoints', *Clinical Microbiology and Infection*, 20(3), pp. 391–408.
Varga, C. *et al.* (2009) 'Associations between reported on-farm antimicrobial use practices and observed antimicrobial resistance in generic fecal Escherichia coli isolated from Alberta finishing swine farms', *Preventive Veterinary Medicine*, 88(3), pp. 185–192. doi: 10.1016/j.prevetmed.2008.10.002.
Vico, J. P. *et al.* (2011) 'Salmonellosis in finishing pigs in Spain: prevalence, antimicrobial agent susceptibilities, and risk factor analysis.', *Journal of food protection*, 74(7), pp. 1070–1078. doi: 10.4315/0362-028X.JFP-10-515.
Vieira, A. R. *et al.* (2009) 'Association between tetracycline consumption and tetracycline resistance in Escherichia coli from healthy Danish slaughter pigs.', *Foodborne pathogens and disease*, 6(1), pp. 99–109.
Vieira, A. R. *et al.* (2011) 'Trends in slaughter pig production and antimicrobial consumption in Danish slaughter pig herds, 2002-2008', *Epidemiology and Infection*, 139(10), pp. 1601–1609.
Vigre, H. *et al.* (2010) 'Use of register data to assess the association between use of antimicrobials and outbreak of Postweaning Multisystemic Wasting Syndrome (PMWS) in Danish pig herds', *Preventive Veterinary Medicine*, 93(2–3), pp. 98–109.
Visschers, V. H. M. *et al.* (2016) 'A Comparison of Pig Farmers' and Veterinarians' Perceptions and Intentions to Reduce Antimicrobial Usage in Six European Countries', *Zoonoses and Public Health*, 63(7), pp. 534–544. doi: 10.1111/zph.12260.
Wales, A. and Davies, R. (2015) 'Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens', *Antibiotics*, 4(4), pp. 567–604. doi: 10.3390/antibiotics4040567.
WHO (2012) The evolving threat of antimicrobial resistance - Options for action, Available at: http://apps.who.int/iris/bitstream/10665/44812/1/9789241503181_eng.pdf (Accessed: 15 January 2018).
WHO (2014) 'Antimicrobial resistance. Global Report on Surveillance', *Bulletin of the World Health Organization*, pp. 383–394. doi: 10.1007/s13312-014-0374-3.
Wielinga, P. R. *et al.* (2014) 'Evidence-based policy for controlling antimicrobial resistance in the food chain in Denmark', *Food Control*, 40(1), pp. 185–192. doi: 10.1016/j.foodcont.2013.11.047.
Wiuff, C. *et al.* (2003) 'The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the

selection of quinolone resistance among Salmonella and coliforms in pigs', *Research in Veterinary Science*, 75(3), pp. 185–193. doi: 10.1016/S0034-5288(03)00112-7.

World Health Organization (2016) 'WHO list of Critically Important Antimicrobials for Human Medicine (WHO CIA list)'. WHO, p. 2. Available at: http://www.who.int/foodsafety/publications/cia2017.pdf?ua=1 (Accessed: 15 January 2018).

Wright, G. D. (2007) 'The antibiotic resistome: the nexus of chemical and genetic diversity', *Nature Reviews Microbiology*, 5(1740–1534 (Electronic)), pp. 175–186. doi: 10.1038/nrmicro1614.

Zhang, L. *et al.* (2013) 'Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota.', *Antimicrobial Agents and Chemotherapy. American Society for Microbiology*, 57(8), pp. 3659–66. doi: 10.1128/AAC.00670-13.

Österberg, J. *et al.* (2016) 'Antibiotic Resistance in Escherichia coli from Pigs in Organic and Conventional Farming in Four European Countries', *PLOS ONE*, 11(6), p. e0157049. doi: 10.1371/journal.pone.0157049.

Appendix A. Additional results to Manuscript I

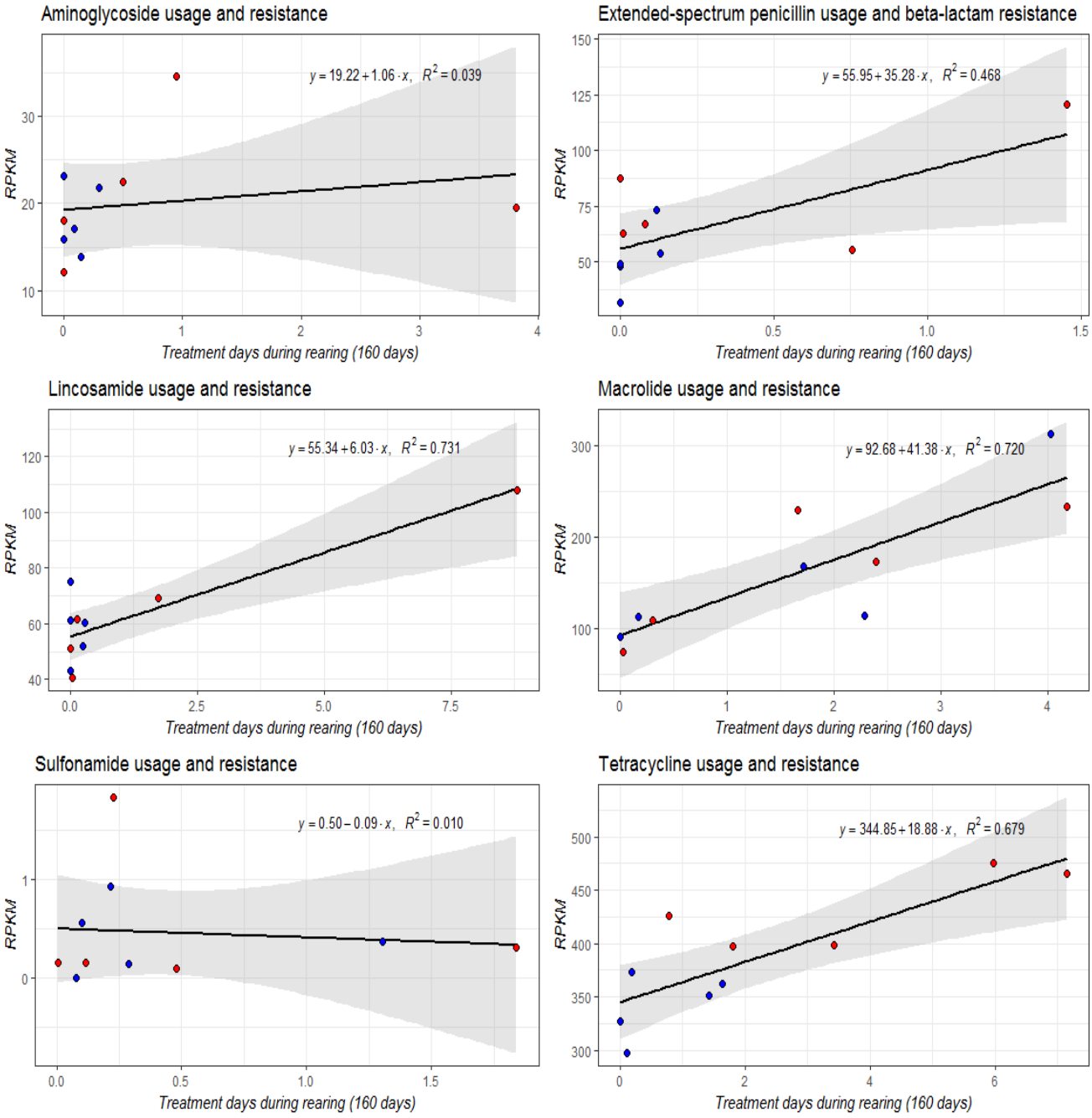


Fig. A1. Univariable linear regression plots (solid line) with 95% confidence interval (grey outline) of WCS – RPKM of the AMR genes of: aminoglycosides, lincosamides, macrolides, beta-lactams, sulfonamides and tetracyclines as a function of treatment days during rearing (160 days) for the AM classes: aminoglycosides, lincosamides, macrolides, extended-spectrum penicillins, sulfonamides and tetracyclines, respectively. The red points denote the initially high users and the blue points depict the initially low users. The function and the R-squared *(R^2)* value are shown in the top left corner of each model.

Appendix B. Additional results to Manuscript II

Table B1. The counted observations, the completeness (Compl.), the correctness (Correct.), the correlation coefficient (**r),** the average adjusted **r**, the Fisher's z estimate, and the average adjusted correlation coefficient through Fisher's z transformation ($\mathbf{r}_z$) at farm, age-group (piglets – sows, weaners and finishers), dispensing-type (parenteral and peroral), and AM-class (aminoglycosides, aminoglycosides combined with narrow-spectrum (nrw.) penicillins, colistin, lincosamides combined with spectinomycin, macrolides, phenicols, narrow-spectrum (nrw.) penicillins, extended-spectrum (ext.) penicillins, sulfonamides combined with trimethoprim (TMP), tetracyclines and tiamulin) level between $Doses_{farm}$ and $Doses_{method.1,}$ $Doses_{method.2.}$ $Doses_{method.3,}$ $Doses_{method.4\ and}$ $Doses_{method.5}$, respectively. In addition, the reliability coefficient ($\boldsymbol{\rho}_{xx}$) without and with average adjustment at farm, age-group, dispensing type, and antimicrobial class level for the $Doses_{method.1,}$ $Doses_{method.2.}$ $Doses_{method.3,}$ $Doses_{method.4\ and}$ $Doses_{method.5.}$

Method 1

	Counts	Comple.	Correct.	r	Fisher's z	r_z	ρ_{xx}
Study population	787	0.597	0.914	0.701	0.868	0.701	0.604
Farm 1	10	0.667	0.857	0.167	0.168		0.063
Farm 2	56	0.623	0.917	0.706	0.878		0.534
Farm 3	20	0.600	1.000	-0.024	-0.025		0.475
Farm 4	109	0.842	0.914	0.898	1.460		0.826
Farm 5	33	0.273	1.000	0.618	0.721		0.456
Farm 6	98	0.404	0.905	0.240	0.245		0.208
Farm 7	24	0.870	0.952	0.390	0.412		0.406
Farm 8	62	0.797	0.940	0.651	0.778		0.549
Farm 9	22	0.588	0.667	-0.188	-0.191		0.403
Farm 10	107	0.867	0.978	0.936	1.706		0.885
Farm 11	36	0.286	0.909	0.868	1.324		0.753
Farm 12	18	0.118	0.667	0.450	0.485		0.403
Farm 13	46	0.486	0.667	0.562	0.635		0.473
Farm 14	72	0.507	0.973	0.870	1.335		0.687
Farm 15	55	0.296	0.941	0.259	0.265		0.376
Farm 16	19	0.706	0.857	0.541	0.605		0.452
Adj.by farm		0.558	0.884	0.496	0.675	0.588	0.497
Sows - Piglets	271	0.743	0.951	0.830	1.187		0.723
Weaners	261	0.551	0.882	0.496	0.544		0.528
Finishers	255	0.485	0.893	0.541	0.606		0.476
Adj. by age-group		0.593	0.909	0.622	0.779	0.652	0.576
Parenteral	585	0.594	0.946	0.868	1.324		0.774
Peroral	202	0.609	0.826	0.378	0.398		0.419
Adj. by dispensing-type		0.601	0.886	0.623	0.861	0.697	0.597
Aminoglycosides	25	0.684	0.684	0.673	0.816		0.525
Aminoglycosides-Penicillins (nrw.)	45	0.568	0.962	0.819	1.155		0.692
Colistin	12	0.800	0.800	0.845	1.239		0.652
Lincosamides	41	0.400	0.941	0.316	0.327		0.337
Lincosamides - Spectinomycin	44	0.619	0.929	0.753	0.980		0.622
Macrolides	102	0.670	0.847	0.541	0.606		0.500
Phenicols	3	0.667	1.000	0.733	0.935		0.386
Penicillins (nrw.)	118	0.513	0.984	0.958	1.921		0.922
Penicillins (ext.)	72	0.786	0.965	0.607	0.705		0.514
Sulfonamides - TMP	82	0.727	0.918	0.851	1.259		0.749
Tetracyclines	203	0.539	0.912	0.531	0.592		0.410
Tiamulin	40	0.487	0.950	0.545	0.611		0.581
Adj. by AM-class		0.622	0.908	0.681	0.929	0.730	0.574

Method 2

	Counts	Comple.	Correct.	**r**	Fisher's z	$\mathbf{r}_z$	$\boldsymbol{\rho}_{xx}$
Study population	814	0.715	0.885	0.747	0.965	0.747	0.654
Farm 1	12	1.000	0.750	0.428	0.457		0.117
Farm 2	61	0.792	0.840	0.727	0.923		0.564
Farm 3	20	0.600	1.000	-0.038	-0.038		0.472
Farm 4	112	0.941	0.896	0.906	1.505		0.844
Farm 5	33	0.394	1.000	0.691	0.851		0.569
Farm 6	101	0.638	0.896	0.286	0.294		0.242
Farm 7	24	0.957	0.957	0.413	0.439		0.425
Farm 8	64	0.847	0.909	0.699	0.865		0.577
Farm 9	22	0.765	0.722	0.387	0.408		0.532
Farm 10	107	0.895	0.979	0.931	1.662		0.875
Farm 11	37	0.429	0.882	0.831	1.192		0.703
Farm 12	22	0.235	0.444	0.278	0.286		0.431
Farm 13	49	0.514	0.613	0.576	0.656		0.500
Farm 14	74	0.634	0.938	0.912	1.537		0.748
Farm 15	56	0.463	0.926	0.753	0.980		0.685
Farm 16	20	0.882	0.833	0.781	1.048		0.671
Adj.by farm		0.687	0.849	0.598	0.817	0.673	0.560
Sows - Piglets	279	0.851	0.925	0.842	1.227		0.739
Weaners	267	0.638	0.866	0.541	0.605		0.560
Finishers	268	0.647	0.852	0.635	0.749		0.559
Adj. by age-group		0.712	0.881	0.672	0.860	0.696	0.619
Parenteral	600	0.693	0.920	0.876	1.357		0.784
Peroral	214	0.788	0.801	0.478	0.520		0.494
Adj. by dispensing-type		0.740	0.861	0.677	0.938	0.734	0.639
Aminoglycosides	27	0.947	0.692	0.867	1.321		0.797
Aminoglycosides-Penicillins (nrw.)	47	0.659	0.906	0.830	1.187		0.709
Colistin	12	0.900	0.818	0.774	1.030		0.592
Lincosamides	44	0.475	0.826	0.358	0.375		0.367
Lincosamides-Spectinomycin	45	0.786	0.917	0.817	1.148		0.729
Macrolides	103	0.769	0.854	0.685	0.839		0.605
Phenicols	3	0.667	1.000	0.733	0.935		0.386
Penicillins (nrw.)	123	0.641	0.926	0.957	1.914		0.921
Penicillins (ext.)	73	0.843	0.952	0.634	0.748		0.537
Sulfa - TMP	83	0.805	0.912	0.859	1.290		0.761
Tetracyclines	211	0.705	0.883	0.536	0.598		0.449
Tiamulin	43	0.538	0.840	0.543	0.609		0.583
Adj. by AM-class		0.728	0.877	0.716	1.000	0.761	0.620

Method 3

	Counts	Comple.	Correct.	**r**	Fisher's z	$\mathbf{r}_z$	$\boldsymbol{\rho}_{xx}$
Study population	827	0.754	0.873	0.761	0.999	0.761	0.673
Farm 1	13	1.000	0.692	0.507	0.558		0.220
Farm 2	61	0.811	0.843	0.727	0.923		0.564
Farm 3	21	0.700	0.933	-0.045	-0.045		0.472
Farm 4	114	0.950	0.881	0.868	1.324		0.795
Farm 5	34	0.515	0.944	0.821	1.158		0.711
Farm 6	102	0.681	0.889	0.287	0.295		0.277
Farm 7	24	1.000	0.958	0.475	0.516		0.459
Farm 8	64	0.847	0.909	0.699	0.865		0.577
Farm 9	23	0.824	0.700	0.625	0.733		0.620
Farm 10	107	0.895	0.979	0.931	1.664		0.876
Farm 11	38	0.657	0.885	0.828	1.183		0.738
Farm 12	26	0.353	0.400	0.348	0.364		0.465
Farm 13	50	0.649	0.649	0.598	0.690		0.526
Farm 14	74	0.634	0.938	0.923	1.607		0.833
Farm 15	56	0.463	0.926	0.753	0.980		0.685
Farm 16	20	0.882	0.833	0.808	1.122		0.711
Adj.by farm		0.741	0.835	0.635	0.871	0.702	0.596
Sows - Piglets	279	0.854	0.925	0.842	1.227		0.739
Weaners	271	0.671	0.853	0.552	0.622		0.570
Finishers	277	0.730	0.830	0.668	0.807		0.602
Adj. by age-group		0.752	0.870	0.687	0.885	0.709	0.637
Parenteral	608	0.730	0.908	0.877	1.363		0.787
Peroral	219	0.832	0.788	0.509	0.561		0.527
Adj. by dispensing-type		0.781	0.848	0.693	0.962	0.745	0.657
Aminoglycosides	27	1.000	0.704	0.919	1.585		0.866
Aminoglycosides-Penicillins (nrw.)	49	0.705	0.861	0.833	1.198		0.715
Colistin	14	0.900	0.692	0.805	1.114		0.707
Lincosamides	47	0.650	0.788	0.377	0.397		0.379
Lincosamides-Spectinomycin	45	0.786	0.917	0.803	1.108		0.718
Macrolides	103	0.824	0.862	0.725	0.918		0.641
Phenicols	3	0.667	1.000	0.733	0.935		0.386
Penicillins (nrw.)	125	0.675	0.908	0.958	1.919		0.922
Penicillins (ext.)	73	0.843	0.952	0.633	0.746		0.536
Sulfonamides - TMP	83	0.818	0.913	0.862	1.303		0.765
Tetracyclines	215	0.751	0.868	0.571	0.649		0.505
Tiamulin	43	0.538	0.840	0.452	0.487		0.548
Adj. by AM-class		0.763	0.859	0.723	1.030	0.774	0.641

Method 4							
	Counts	Comple.	Correct.	**r**	Fisher's z	$\mathbf{r}_z$	$\boldsymbol{\rho}_{xx}$
Study population	854	0.834	0.851	0.765	1.009	0.765	0.680
Farm 1	12	1.000	0.750	0.477	0.519		0.116
Farm 2	61	0.981	0.867	0.738	0.946		0.581
Farm 3	21	0.850	0.944	0.061	0.061		0.489
Farm 4	126	0.980	0.798	0.857	1.283		0.787
Farm 5	33	0.606	1.000	0.818	1.150		0.709
Farm 6	103	0.723	0.883	0.327	0.339		0.293
Farm 7	24	0.957	0.957	0.413	0.439		0.425
Farm 8	67	1.000	0.881	0.667	0.806		0.561
Farm 9	27	0.941	0.615	0.641	0.760		0.627
Farm 10	107	0.962	0.981	0.928	1.640		0.870
Farm 11	39	0.571	0.833	0.830	1.187		0.739
Farm 12	20	0.176	0.500	0.321	0.332		0.454
Farm 13	58	0.649	0.533	0.694	0.855		0.620
Farm 14	74	0.915	0.956	0.933	1.681		0.856
Farm 15	58	0.556	0.882	0.769	1.017		0.701
Farm 16	24	0.941	0.696	0.826	1.175		0.731
Adj.by farm		0.801	0.817	0.644	0.887	0.710	0.597
Sows - Piglets	284	0.969	0.917	0.853	1.268		0.757
Weaners	291	0.782	0.798	0.541	0.606		0.575
Finishers	279	0.739	0.824	0.666	0.803		0.598
Adj. by age-group		0.830	0.846	0.687	0.892	0.713	0.644
Parenteral	619	0.818	0.897	0.885	1.397		0.800
Peroral	235	0.883	0.738	0.519	0.575		0.536
Adj. by dispensing-type		0.850	0.818	0.702	0.986	0.756	0.668
Aminoglycosides	25	1.000	0.760	0.918	1.574		0.864
Aminoglycosides-Penicillins (nrw.)	51	0.932	0.854	0.919	1.584		0.858
Colistin	16	0.900	0.600	0.832	1.194		0.733
Lincosamides	46	0.600	0.800	0.776	1.035		0.710
Lincosamides-Spectinomycin	43	0.976	0.976	0.917	1.570		0.863
Macrolides	108	0.835	0.817	0.689	0.845		0.612
Phenicols	3	0.333	1.000	0.155	0.156		0.180
Penicillins (nrw.)	130	0.778	0.875	0.970	2.092		0.943
Penicillins (ext.)	75	0.914	0.928	0.640	0.759		0.549
Sulfonamides - TMP	83	0.909	0.921	0.876	1.360		0.789
Tetracyclines	217	0.788	0.864	0.570	0.648		0.501
Tiamulin	57	0.846	0.647	0.532	0.593		0.581
Adj. by AM-class		0.818	0.837	0.733	1.118	0.807	0.682

Method 5							
	Counts	Comple.	Correct.	**r**	Fisher's z	$\mathbf{r}_z$	$\boldsymbol{\rho}_{xx}$
Study population	855	0.859	0.853	0.765	1.009	0.765	0.682
Farm 1	11	0.889	0.800	0.696	0.860		0.200
Farm 2	61	0.981	0.867	0.738	0.946		0.581
Farm 3	21	0.900	0.947	0.029	0.029		0.486
Farm 4	126	1.000	0.802	0.851	1.261		0.781
Farm 5	34	0.667	0.957	0.820	1.156		0.712
Farm 6	103	0.777	0.890	0.321	0.333		0.302
Farm 7	24	1.000	0.958	0.475	0.516		0.459
Farm 8	67	1.000	0.881	0.667	0.806		0.561
Farm 9	25	1.000	0.680	0.610	0.709		0.604
Farm 10	107	0.971	0.981	0.927	1.640		0.870
Farm 11	39	0.571	0.833	0.830	1.187		0.739
Farm 12	21	0.235	0.500	0.326	0.339		0.456
Farm 13	60	0.811	0.566	0.738	0.946		0.661
Farm 14	74	0.915	0.956	0.933	1.681		0.856
Farm 15	58	0.556	0.882	0.769	1.017		0.701
Farm 16	24	0.941	0.696	0.826	1.175		0.731
Adj.by farm		0.826	0.825	0.660	0.913	0.722	0.606
Sows - Piglets	284	0.969	0.917	0.853	1.268		0.757
Weaners	293	0.823	0.800	0.544	0.609		0.578
Finishers	278	0.776	0.835	0.662	0.797		0.600
Adj. by age-group		0.856	0.850	0.686	0.891	0.712	0.645
Parenteral	620	0.846	0.899	0.886	1.404		0.802
Peroral	235	0.899	0.742	0.514	0.568		0.536
Adj. by dispensing-type		0.873	0.820	0.700	0.986	0.756	0.669
Aminoglycosides	25	1.000	0.760	0.918	1.574		0.864
Aminoglycosides-Penicillins (nrw.)	51	0.932	0.854	0.919	1.584		0.858
Colistin	16	0.900	0.600	0.832	1.194		0.733
Lincosamides	46	0.600	0.800	0.776	1.035		0.710
Lincosamides-Spectinomycin	43	0.976	0.976	0.917	1.570		0.863
Macrolides	109	0.890	0.818	0.676	0.821		0.605
Phenicols	3	0.333	1.000	0.155	0.156		0.180
Penicillins (nrw.)	130	0.778	0.875	0.970	2.092		0.943
Penicillins (ext.)	75	0.914	0.928	0.640	0.759		0.549
Sulfonamides - TMP	83	0.935	0.923	0.885	1.396		0.801
Tetracyclines	220	0.850	0.859	0.579	0.661		0.512
Tiamulin	54	0.846	0.688	0.526	0.585		0.579
Adj. by AM-class		0.830	0.840	0.733	1.119	0.807	0.683

Table B2. Treatment age-group instructed by the vets compared to the recorded age-group in VetStat of the AM-classes; aminoglycosides, aminoglycosides combined with narrow-spectrum (nrw.) penicillins, colistin, lincosamides, lincosamides combined with spectinomycin, macrolides, extended-spectrum (ext.) penicillins, narrow-spectrum (nrw.) penicillins, sulfonamides combined with trimethoprim (TMP), tetracyclines and tiamulin, used in the study.

AM-class	Dispensering	Vet-instruction	VetStat-record	Count
Aminoglycosides	Peroral	Piglet	Sow	17
Aminoglycosides - Penicillins (nwr.)	Parenteral	Piglet	Sow	22
Aminoglycosides - Penicillins (nwr.)	Parenteral	Weaner	Sow	1
Aminoglycosides - Penicillins (nwr.)	Parenteral	Weaner	Weaner	1
Colistin	Peroral	Piglet	Sow	1
Colistin	Peroral	Weaner	Weaner	5
Lincosamides	Parenteral	Sow	Sow	7
Lincosamides	Parenteral	Finisher	Finisher	6
Lincosamides - spectinomycin	Parenteral	Piglet	Sow	8
Lincosamides - spectinomycin	Peroral	Weaner	Weaner	8
Macrolides	Parenteral	Sow	Sow	8
Macrolides	Parenteral	Weaner	Weaner	7
Macrolides	Parenteral	Finisher	Finisher	9
Macrolides	Parenteral_LA	Piglet	Sow	20
Macrolides	Parenteral_LA	Weaner	Weaner	2
Macrolides	Peroral	Weaner	Weaner	7
Macrolides	Peroral	Finisher	Finisher	6
Penicillins (nwr.)	Parenteral	Sow	Sow	15
Penicillins (nwr.)	Parenteral	Weaner	Weaner	9
Penicillins (nwr.)	Parenteral	Finisher	Finisher	28
Penicillins (ext.)	Parenteral	Finisher	Finisher	1
Penicillins (ext.)	Parenteral_LA	Piglet	Sow	28
Penicillins (ext.)	Parenteral_LA	Sow	Sow	11
Penicillins (ext.)	Peroral	Weaner	Weaner	7
Sulfonamides -TMP	Parenteral	Piglet	Sow	12
Sulfonamides -TMP	Parenteral	Piglet	Weaner	6
Sulfonamides -TMP	Parenteral	Sow	Sow	28
Sulfonamides -TMP	Parenteral	Weaner	Weaner	7
Sulfonamides -TMP	Parenteral	Finisher	Finisher	2
Sulfonamides -TMP	Peroral	Sow	Sow	1
Sulfonamides -TMP	Peroral	Weaner	Weaner	5
Tetracyclines	Parenteral	Sow	Sow	14
Tetracyclines	Parenteral	Weaner	Sow	1
Tetracyclines	Parenteral	Weaner	Weaner	13
Tetracyclines	Parenteral	Finisher	Finisher	21
Tetracyclines	Parenteral_LA	Weaner	Weaner	8
Tetracyclines	Parenteral_LA	Finisher	Weaner	1
Tetracyclines	Parenteral_LA	Finisher	Finisher	4

Tetracyclines	Peroral	Weaner	Weaner	19
Tetracyclines	Peroral	Finisher	Finisher	12
Tiamulin	Parenteral	Sow	Sow	9
Tiamulin	Parenteral	Finisher	Finisher	1
Tiamulin	Peroral	Weaner	Weaner	3
Tiamulin	Peroral	Weaner	Finisher	7
Total				408

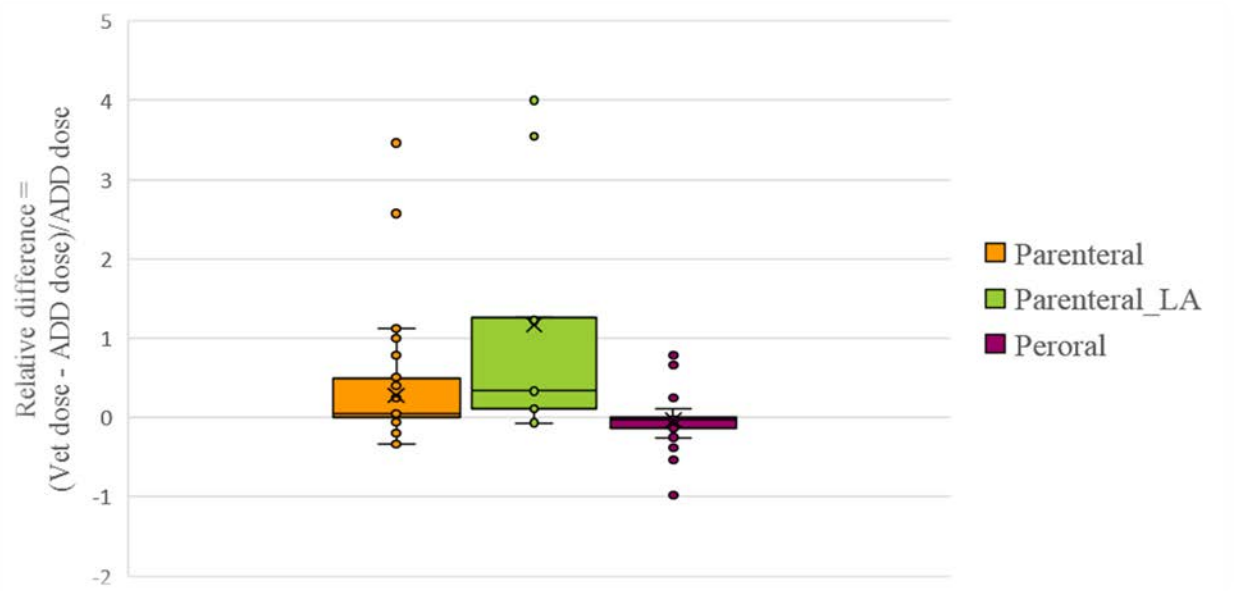


Fig. B3. Relative error between the treatment dose instructed by the vets and the standard ADD of parenteral, parenteral long-acting (LA) and peroral antimicrobials. Four observation (parenteral_LA (#3) and parenteral (#1)) were removed because the relative difference was more than 9 times higher compared to the standard ADD, thus assumed to be writing error.

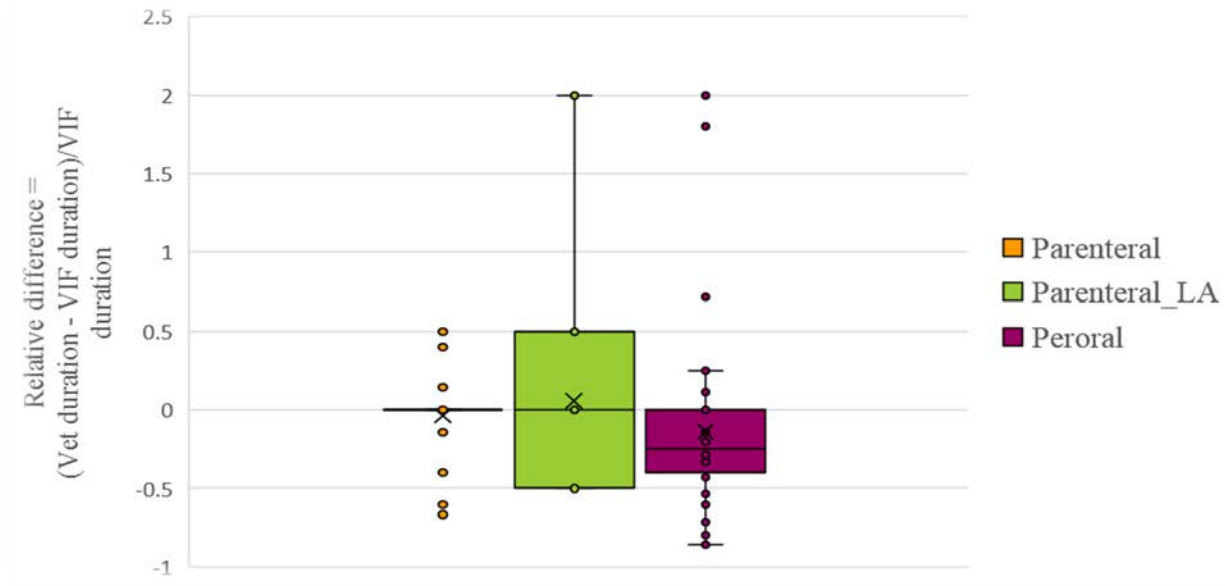


Fig. B4. Relative error between the treatment duration instructed by the vets and the standard treatment duration from the medicine catalogue of the Danish Veterinary Medical Industry of parenteral, parenteral long-acting (LA) and peroral.

Table B5. Overview of the antimicrobial classes, dispensing types and diseases registered in the vet instructions

Antimicrobial class	Dispensing	Disease
Aminoglycosides	Peroral	Coli-diarrhea, Diarrhea
Aminoglycosides-Penicillins (nrw.)	Parenteral	Arthritis
Colistin	Peroral	Coli-diarrhea, Diarrhea
Lincosamides	Parenteral	Arthritis, Mycoplasma
Lincosamides-Spectinomycin	Parenteral	Diarrhea
	Peroral	Diarrhea, Lawsonia, Post-weaning enteritis
Macrolides	Parenteral	Diarrhea, Lawsonia, Mastitis, Pasteurella, Pneumonia
	Parenteral_LA	Mycoplasma, Pasteurella, Pneumonia
	Peroral	Diarrhea, Glassers disease, Lawsonia
Penicillins (nrw.)	Parenteral	Arthritis, Meningitis, MMA (mastitis/metritis/agalactia), Pneumonia, Ulcer
Penicillins (ext.)	Parenteral	Ear inflammation
	Parenteral_LA	Arthritis, MMA (mastitis/metritis/agalactia), Omphalitis
	Peroral	Arthritis, Coli-diarrhea, Meningitis, Pasteurella
Sulfonamides-Trimethoprim	Parenteral	Coli-diarrhea, Diarrhea, Meningitis, MMA (mastitis/metritis/agalactia), Post-weaning enteritis
	Peroral	Arthritis, Pneumonia, Metritis
Tetracyclines	Parenteral	Abscess, Arthritis, Diarrhea, Hoof abscess, Lawsonia, Regional enteritis, Ulcer
	Parenteral_LA	Arthritis, Lawsonia, Pneumonia
	Peroral	Lawsonia, Pneumonia , Regional enteritis
Pleuromutilins	Parenteral	Arthritis, Mycoplasma
	Peroral	Diarrhea, Lawsonia, Mycoplasma, Regional enteritis, Pneumonia

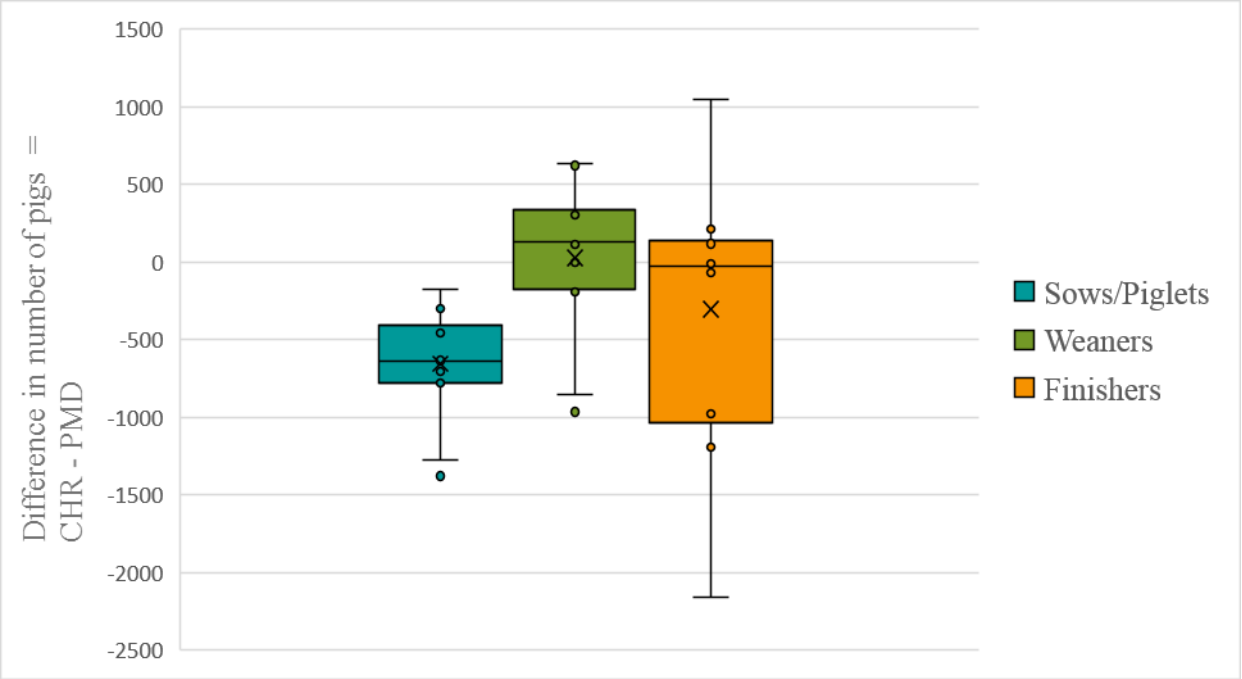


Fig. B6. Differences in number of pigs by comparing the number of sows, weaners and finishers registered in the CHR with the number of piglet, weaners and finisher registered in the PMD (production).